

Mapping NAD⁺ metabolism in the brain of ageing Wistar rats: potential targets for influencing brain senescence

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Abstract Over the last decade, the importance of NAD⁺ has expanded beyond its role as an essential cofactor for energy metabolism. NAD⁺ has emerged as a major signalling molecule that serves as the sole substrate for several enzymatic reactions including the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP), NAD-dependent protein deacetylases or CD38, and transcriptional factors by a new class of histone deacetylases known as sirtuins. NAD⁺ levels are regulated by the metabolic status and cellular stress caused by oxidative stress and DNA damage. Since a detailed study of NAD⁺ metabolism in the healthy ageing mammalian brain is nascent, we examined the

effect of ageing on intracellular NAD⁺ metabolism in different brain regions in female Wistar rats in young (3 months), middle aged (12 months) and older adults (24 months). Our results are the first to show a significant decline in intracellular NAD⁺ levels and NAD:NADH ratio with ageing in the CNS, occurring in parallel to an increase in lipid peroxidation and protein oxidation (*o*- and *m*-tyrosine) and a decline in total antioxidant capacity. Hyperphosphorylation of H2AX levels was also observed together with increased PARP-1 and PARP-2 expression, and CD38 activity, concomitantly with reduced NAD⁺ and ATP levels and SIRT1 function in the cortex,

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brainstem, hippocampus and cerebellum. Reduced activity of mitochondrial complex I–IV and impaired maximum mitochondrial respiration rate were also observed in the ageing rat brain. Among the multiple physiological pathways associated with NAD⁺ catabolism, our discovery of CD38 as the major regulator of cellular NAD⁺ levels in rat neurons indicates that CD38 is a promising therapeutic target for the treatment of age-related neurodegenerative diseases.

Keywords NAD⁺ · Ageing · Oxidative stress · DNA damage · PARP

Introduction

An increasing lifespan in most developed and many developing societies has meant that many individuals will attain an age at which Alzheimer's, Parkinson's and other neurodegenerative disorders become more prevalent (Mattson 2004; Finkel 2005). In the early 1950s, Harman proposed the “free radical theory of ageing”, which postulated that damage to tissues and cells was caused by increased free radical production in aerobic organisms and is a major determining factor for lifespan (Harman 1956). Subsequently, it was found that reactive oxygen species, not all of which are free radicals (since they are not composed of an unpaired electron in the outer shell), may contribute to oxidative cellular damage. A modern version of this tenet suggests that increased ROS is associated with ageing, and age-related degeneration, and increased mortality (Ashok and Ali 1999; Harman 2003; Li and Holbrook 2003; Balaban et al. 2005).

Mitochondrial metabolism is the primary source of cellular ROS in the human body (Kamata and Hirata 1999). The reduction of oxygen to water, which is the key energy producing biological reaction, is associated with the production of highly reactive superoxide anions (Kamata and Hirata 1999; Floyd and Hensley 2002). The brain has the highest level of O₂ consumption per tissue weight and as such generates the highest levels of ROS (Floyd and Hensley 2002). Brain metabolism is reduced in Alzheimer's disease (AD), and increased ROS levels have been associated with extracellular amyloid beta (Aβ), redox-active transition metals, decreased metabolic regulation, and mitochondrial abnormalities (Miranda et al. 2000;

Butterfield et al. 2001; Butterfield 2002). Intracellular ROS, due to their high reactivity, can interact with a spectrum of biological molecules, leading to the oxidation of several macromolecules, such as proteins, lipids, and nucleic acids (Chen et al. 1995).

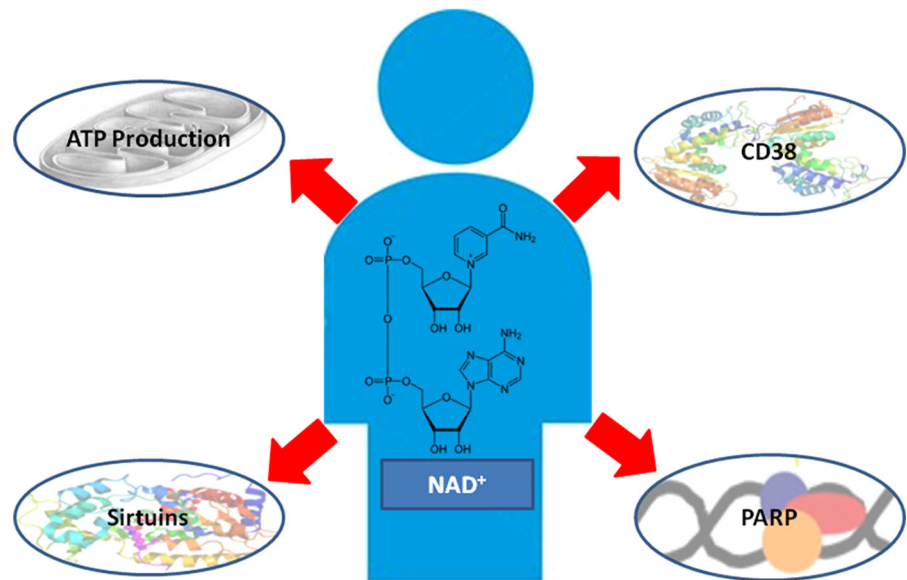
Increased oxidative DNA damage is a major facet associated with the development and progression of age-related degenerative disorders (Bohr 2002). DNA damage may affect the expression of a variety of genes involved in the regulation of cell proliferation or inhibit expression of other genes associated with DNA repair (Bohr 2002).

A large number of physiological protection and repair systems, such as the activation of the DNA nick sensor poly(ADP-ribose) polymerase-1 (PARP) and the antioxidant defence system, are activated by the cell to counteract free radical induced damage, and thereby maintain macromolecular functions (de Marcia 1994; Retz et al. 1998). PARP is an abundant protein-modifying nuclear enzyme that normally functions in DNA repair (Berger et al. 2007). Activation of PARP can repair damaged DNA using NAD⁺ as the essential substrate (Virag et al. 1998; Burkle 2005). However, hyper-activation of PARP can reduce genomic stability and promote cell death (Erdelyi et al. 2005). Importantly, over-activation of PARP is known to precede the death of neuronal cells in AD (Love et al. 1999; Infante et al. 2007) though its impact on NAD⁺ levels in AD has not been previously reported. Of the PARP family of enzymes, PARP-1 and PARP-2 are the only known members whose activity is stimulated by DNA strand breaks (Farres et al. 2013; Kraus and Hottiger 2013; Kutuzov et al. 2013). PARP-1 and PARP-2 induce chromatin decondensation at the damage site leading to initiation of repair mechanisms, and further DNA damage (Bai and Canto 2012).

Apart from its role in the maintenance of DNA repair, NAD⁺ has emerged as an important mediator of several signalling pathways implicated in ageing and neurodegenerative processes. Two major signalling roles of NAD⁺ include the generation of secondary messengers such as cyclic-ADP-ribose (cADPR) following CD38-mediated hydrolysis of NAD⁺, and its role as a substrate for NAD-dependent sirtuin deacetylases (Escande et al. 2013) (Fig. 1). Both signalling pathways have been associated with cellular mechanisms of ageing, metabolic function, transcriptional regulation, cellular stress resistance mechanisms and cell death (Moss et al. 2013; Nipp et al.

Fig. 1 Biological functions associated with NAD⁺.

Apart from its role in oxidative phosphorylation and ATP production, NAD⁺ also serves as the sole substrate for a number of important enzymes including the DNA repair enzyme, PARP, histone deacetylation by sirtuin enzymes associated with transcriptional regulation, ageing and longevity, and CD38, the main generator of the secondary messenger molecule, cADPR



2013; Quarona et al. 2013; Sobko et al. 2013; Xiong et al. 2013; Xu et al. 2013). Numerous studies have postulated the potential role of CD38, a type II membrane protein, as the major NADase in mammalian cells. CD38 cleaves NAD⁺ and nicotinamide adenine dinucleotide phosphate (NADP⁺), leading to the formation of cyclic ADP ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP), and ADP-ribose (ADPR) (Aarhus et al. 1995; Chidambaram and Chang 1999; Torti et al. 1999). These reaction products are involved in the stimulation of intracellular calcium [Ca²⁺]_i release. CD38 regulates numerous processes, including cell activation and proliferation, muscle contraction, hormone release, and cell immunity (Lopatina et al. 2012). Impaired CD38 function is associated with deleterious effects on immunity, metabolic dysfunction, and behavioral deficits in mice (Burkle 2005; Burkle et al. 2004; Butterfield 2002; Butterfield et al. 2001; Canevari et al. 1999; Chen et al. 1995, 2013; Chidambaram and Chang 1999). Tissue levels of NAD⁺ were found to be tenfold to 20-fold higher in CD38 deficient mice compared to wild-type animals. In addition, NADase activity was almost absent in CD38 knockout mice (Aksoy et al. 2006a, b; Savaskan et al. 2006).

Sirtuins play an important role in promoting cell viability under conditions of oxidative stress and genotoxic insult via caloric restriction regimens (Anderson and Maes 2013; Bagul and Banerjee 2013; Merksamer et al. 2013; Nadal-Serrano et al.

2013; Shinmura 2013). SIRT1, the founding member of the sirtuin histone deacetylase family has been shown to reduce age-associated pathologies, including insulin resistance, cardiovascular diseases and neurodegenerative diseases. The profound impact of sirtuins in maintaining longevity and neuroprotection suggests that it may be considered a potential therapeutic target for the treatment of age-related degenerative disorders (Braidly et al. 2012; Massudi et al. 2012). Therefore, promotion of cellular NAD⁺ anabolism is crucial to the success of this process (Braidly et al. 2008). However, to date, no study has published on the regulatory role of CD38 on intracellular NAD⁺ levels, and PARP and SIRT1 activities in the ageing rat brain.

Materials and methods

Reagents and Chemicals

Phosphate buffer solution (PBS) was from Invitrogen (Melbourne, Australia). Nicotinamide, bicine, β-nicotinamide adenine dinucleotide reduced form (β-NADH), 3-[-4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), alcohol dehydrogenase (ADH), sodium pyruvate, TRIS, γ-globulins, *N*-(1-naphthyl) ethylenediamine dihydrochloride, EGTA, EDTA, tricarboxylic acid (TCA), Hepes, proteinase K, Percoll, mannitol, 1,-dithio DL-threitol (DTT), KCN, decylubiquinone, succinate, antimycin,

rotenone, cytochrome *c*, and sodium borohydride were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate (PMS) was obtained from ICN Biochemicals (Cleveland, Ohio). Bradford reagent was obtained from BioRad (Hercules, CA, USA). DAPI and polyclonal antibodies (pAb) for β -actin and all chemicals used for Western blots (unless otherwise stated) were obtained from Sigma-Aldrich (Castle-Hill, Australia). Polyclonal antibodies for detection of (E)-4-hydroxynonenal, phospho-H2AX-ser139 were obtained from Alexis Biochemicals (San Diego, CA). Monoclonal antibody for the detection of poly(ADP-ribose) was purchased from Alexis Biochemicals (San Diego, CA). Alexa 488- or Alexa 594-conjugated anti-mouse IgG or anti-rabbit were purchased from Invitrogen (Sydney, Australia). All commercial antibodies were used at the concentrations recommended by the manufacturer.

Animals

Female Wistar rats were used in the following age groups: 3 months (equivalent to a young human adult aged 20 years), 12 months (equivalent to a middle-aged human aged 40 years) and 24 months (equivalent to an aged human greater than 80 years) (Collier and Coleman 1991). The animals were individually housed in an environmentally controlled room under a 12 h light/dark cycle at 23 °C and were fed commercial rat chow and water ad libitum. All experiments were performed with approval from the Animal Ethics Committee of the University of Sydney. Anaesthesia was induced with a mixture of O₂, nitrous oxide (N₂O), and 5 % halothane, followed by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). Transcardial perfusion was performed using 0.1 M PBS, pH 7.4. Whole brain was removed, washed with DPBS and used immediately for a variety of biochemical and histochemical procedures.

Assay of *o*- and *m*-tyrosine for the measurement of protein carbonyl formation

The assay of *o*- and *m*-tyrosine was performed as previously described (Poljak et al. 2000). Briefly, calibration standards of phenylalanine (313 pmol–50 nmol) and *o*- and *m*-tyrosine (313 fmol–50 pmol) were used for quantification. Labelled [¹³C₆]-phenylalanine was used as an internal standard and was added to all samples and

standards. The cortex, hippocampus, cerebellum and brainstem were carefully cut from the whole brain, homogenised, and placed in tapered glass vials. The protein was then allowed to precipitate (~18 h, –20 °C) following the addition of 10 volumes of acetone/HCl (100:1, v:v). The precipitate was centrifuged (14,000 g, 30 min, ambient temperature) and the supernatant discarded. Internal standard (10 μ l, 1 mg/ml [¹³C₆]-phenylalanine) containing trace quantities of [¹³C₆]-tyrosine, was added to all samples and standards, and dried under reduced pressure (<1 Torr, 1–2 h). Gas phase acid hydrolysis was then performed (150 °C, 1 h). Samples and standards were then derivatised using a three-step derivatisation approach, using heptafluorobutyric anhydride (HFBA), pentafluorobenzylbromide (PFBBBr) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), vacuum dried for 10 min, and reconstituted in 50 μ l ethyl acetate. Each standard and sample (1 μ l) was injected into the GC/MS using pressure pulse splitless loading (6890 N gas chromatograph interfaced to a 5,973 mass selective detector from Agilent technologies (Ryde, Australia), with methane as the ECNI reagent gas. Chromatography was performed on a fused silica capillary column (HP-5MS; 30 m x 0.25 mm i.d. x 0.25 μ m, film thickness), with helium carrier gas and using the following chromatographic parameters: inlet temperature 250 °C, transfer line temperature 280 °C, programmed oven temperature gradient with initial temperature of 150 °C for 3 min, then a temperature ramp of 30 °C/min to 290 °C and a final time of 3 min at 290 °C. Final *o*- and *m*-tyrosine levels were expressed relative to phenylalanine levels using peak area integration.

Western blots for the detection of 4-hydroxynonenal and, poly(ADP)-ribose polymerase-1 (PARP-1), and poly(ADP)-ribose polymerase-2 (PARP-2)

The cortex, hippocampus, cerebellum and brainstem were carefully dissected from the whole brain and homogenised in RIPA lysis buffer containing 50 mM Tris-HCl (pH7.4); IGEPAL 1; 0.25 % Na-deoxycholate; 1 mM EDTA, 150 mM NaCl; 1 μ g/ml each of the protease inhibitors aprotinin, leupeptin and pepstatin; 1 mM Na₃VO₄; and 1 mM NaF. After 1 h, the protein extract was obtained by centrifuging at 14,000 g, for 30 min at 4 °C. Equal amounts of protein extract (30 μ g) were dissolved in Laemmli sample buffer

Table 1 List of primary antibodies used

Antibody	Type	Antigen	Dilution	Source
(E)-4-Hydroxynonenal	Polyclonal	Free HNE (E)-4-hydroxynonenal	1:1,000	Alexis Biochemicals
Phospho-H2AX-Serine139	Polyclonal	Phosphorylated H2AX at Serine 139	1:1,000	Alexis Biochemicals
Poly(ADP-ribose) (10H)	Monoclonal	Poly(ADP-ribose)	1:400	Alexis Biochemicals
CD38 antibody	Monoclonal	CD38	1:1,000	Alexis Biochemicals
Poly(ADP-ribose) polymerase 1 antibody	Monoclonal	Poly(ADP-ribose) polymerase-1	1:1,000	Sigma-Aldrich
Poly(ADP-ribose) polymerase 2 antibody	Monoclonal	Poly(ADP-ribose) polymerase-2		

(Hercules, CA), boiled for 5 min, electrophoresed using BioRad precast 8–12 % (v/v) polyacrylamide SDS-PAGE gels (Hercules, CA), and electrotransferred onto BioRad PVDF membranes (Hercules, CA). Membranes were blocked with 5 % non-fat milk dissolved in TBS for 1 h and incubated with primary antibody (Table 1) overnight at 4 °C. After incubation with primary antibodies, membranes were washed in TBS-Tween-20 and incubated with Sigma HRP conjugated secondary antibodies (Castle Hill, Australia) for 1 h at ambient temperature. After further washing in TBS-Tween-20, the membranes were incubated with an ECL plus reagent (RPN2132, Amersham) and protein bands were visualised on X-ray films. The bands were quantified by integration of pixel intensity using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland), and normalised to β -actin, which served as an internal control.

Total antioxidant capacity assay

The total antioxidant capacity in the ageing brain tissue was evaluated with the Trolox equivalent antioxidant capacity assay using a standard Cayman's antioxidant assay kit (Ann Arbor, Michigan). Briefly, the cortex, hippocampus, cerebellum and brainstem were carefully dissected from the whole brain and homogenised on ice in a buffer containing 5 mM potassium phosphate; 0.9 % NaCl; and 0.1 % glucose, pH 7.4. the samples centrifuged (10,000 g, 15 min, 4 °C), and the supernatant was collected for the assay. The sample (10 μ l) was added to metmyoglobin (10 μ l) and chromogen (150 μ l). The reaction was initiated by adding 40 μ l of hydrogen peroxide (440 μ M) and the samples incubated on a shaker (5 min, ambient temperature). The absorbance was

read at 405 nm using a BioRad Model 680XR microplate reader (Hercules, CA). Sample concentrations were determined using a standard curve constructed using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly employed as an antioxidant. The results were expressed as mmol Trolox/L/mg protein.

DNA damage assay

Since H2AX phosphorylation at serine 139 is a double strand DNA break sensor, although single strand breaks can progress to double strand DNA breaks by multiple mechanisms. This makes phosphor-H2AX a sensitive marker for oxidative DNA damage (Huang et al. 2006). We measured the phosphorylation of H2AX at serine 139 using a DNA damage assay kit purchased from Active Motif (Carlsbad, CA). Briefly, the cortex, hippocampus, cerebellum and brainstem were carefully dissected from the whole brain and suspended on ice in a buffer containing 1 mM PMSF and centrifuged (10,000 $\times$ g, 4 min, 4 °C). Afterwards 200 μ l of warm SDS lysis buffer containing 1 μ g/ml each of aprotinin, leupeptin and pepstatin and 1 mM PMSF were then added and incubated for 10 min on ice. All samples were sonicated to generate 100–1,000-bp DNA fragments. Anti-phospho-H2AX-ser139 (5 μ g/ml) was added to the lysate with cold DPBS and incubated overnight at 4 °C. The primary antibody was removed by washing the lysate 3 times with 200 μ l DPBS. Alexis 488 goat anti-rabbit IgG secondary antibody (1:1,000) was added to the lysate and incubated for 1 h at ambient temperature. The secondary antibody was removed by washing the lysate twice with 200 μ l DPBS. The fluorescence was read using a Fluostar Optima

Fluorometer (New York, NY). Filter excitation and emission was set at 485 and 520 nm respectively.

Isolation and extraction of nuclei

Aliquots of brain homogenate from the cortex, hippocampus, cerebellum and brain stem (with protease inhibitors) were centrifuged through 4 ml of 30 % sucrose solution containing 10 mM Tris HCl (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$ at 1,300 g for 10 min at 4 °C. The pellet was washed with cold 10 mM Tris–HCl (pH 7.4) and 10 mM NaCl. The nuclei were later suspended in 50–100 μ l extraction buffer containing 50 mM Hepes KOH (pH 7.4), 420 mM NaCl, 0.5 mM EDTA, 0.1 mM EGTA, and glycerol 10 %, sonicated for 30 s, and allowed to incubate on ice for 30 min followed by centrifugation (10,000 $\times$ g, 10 min). The nuclear extract were stored at –80 °C till required for assay.

CD38 activity assay

NADase activity was measured in lysates from the cortex, hippocampus, cerebellum and brainstem as described previously (Hara-Yokoyama et al. 1996) and based on the radioactivities of NAD^+ and nicotinamide using UV light. The ADP ribosyl cyclase activity of CD38 was measured in these lysates using a fluorescence based assay as previously described (Sieck et al. 2008). The assay is based on the fact ADP ribosyl cyclase can cyclize the NAD^+ analog, NGD to form the non-hydrolysable fluorescent product, cGDPR. The samples were mixed well and incubated for 10 min at room temperature and the fluorescence intensity (ex. 305 nm, em. 410 nm) was measured every 30 s for a total of 60 min immediately after the addition of NGD using Fluostar Optima Fluorometer (NY, USA) and normalised by the protein content.

SIRT1 deacetylase activity

SIRT1 deacetylase activity was evaluated in nuclear extracts from cortex, hippocampus, cerebellum and brainstem of young, middle-aged, and aged rats, using the Cyclex SIRT1/Sir2 deacetylase fluorometric assay kit (CycLex, Nagano, Japan). The final reaction mixture (100 μ l) contained 50 mM Tris–HCl (pH 8.8), 4 mM $MgCl_2$, 0.5 mM DTT, 0.25 mA/ml

lysyl endopeptidase, 1 μ M Trichostatin A, 200 μ M NAD^+ , and 5 μ l of nuclear sample. The samples were mixed well and incubated for 10 min at room temperature and the fluorescence intensity (ex. 340 nm, em. 460 nm) was measured every 30 s for a total of 60 min immediately after the addition of fluorosubstrate peptide (20 μ M final concentration) using Fluostar optima fluorometer (NY, USA) and normalised by the protein content. The results are reported as relative fluorescence/ μ g of protein (AU).

Measurement of intracellular NAD^+ /NADH levels

Total intracellular NAD ($NADH + NAD^+$) concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky and Swan (1973) adapted for 96 well plate format by Grant and Kapoor (1998) (Bernofsky and Swan 1973; Grant and Kapoor 1998). Briefly, each assay contained 100 mM bicine, pH 7.8; 0.42 mM MTT, and 1.66 mM PMS. For NAD^+ measurement, 20 μ l of ADH in 0.15 % ethanol was added to the reaction mixture. The amount of NAD and NADH were measured as the change in absorbance at 590 nm at 37 °C for 10 min. A BioRad Model 680XR microplate reader (Hercules, CA) was used. The ratio of NAD^+ /NADH was calculated based on results of NAD^+ and NADH concentrations.

Measurement of ATP levels

Intracellular ATP levels were measured in the brain tissue using luciferin-luciferase assay as described previously (Harper et al. 1998). Briefly, tissue were homogenised in 250 μ l extraction solution containing 10 mM KH_2PO_4 , 4 mM $MgSO_4$, pH 7.4), and heated at 98 °C for 4 min and then placed on ice. Afterwards, 50 μ l of sample was added to 100 μ l reaction solution containing 50 mM $NaAsO_2$, 20 mM $MgSO_4$, pH 7.4, and 800 μ g luciferin/luciferase enzyme. The luminosity was measured at 259 nm with an extinction coefficient of 15,400 using a Turner Designs TD 20/20 luminometer (Stratec Biomedical Systems, Germany).

Isolation of mitochondria

All procedures were carried out at 0–4 °C. Briefly, the cortex, hippocampus, cerebellum, and brainstem were

excised from the whole rat brain, washed with DPBS, and treated with proteinase K (1 mg/ml) for 30 s, washed with buffer A (250 mM mannitol; 0.5 mM EGTA; 5 mM Hepes; and fatty acid free bovine serum albumin 0.1 %, pH 7.4 at 4 °C). The samples were homogenised in buffer A (200 µl) using a Teflon pestle. The homogenate was centrifuged twice (600×g, 5 min, 4 °C) and the supernatants combined and further centrifuged (10,300 g, 10 min, 4 °C). The mitochondrial pellets were suspended in 0.5 ml buffer A and transferred to ultracentrifuge tubes containing 1.4 ml buffer B (225 mM mannitol; 1 mM EGTA; 25 mM Hepes; and fatty acid free bovine serum albumin (BSA) 0.1 %, pH 7.4 at 4 °C) and 0.6 ml Percoll. The mixture was centrifuged (105,000×g, 30 min, 4 °C). The pure mitochondrial fraction was purified by washing twice with buffer A (100 µl and centrifugation (10,300×g, 10 min, 4 °C) to remove the Percoll. The mitochondrial extract was stored at –80 °C till required for assay.

Determination of mitochondrial complexes I, II, III, and IV activities

To determine the complex I activity, mitochondrial fractions (0.6 mg/ml) were incubated for 3 min in buffer solution containing 250 mM sucrose; 50 mM potassium-phosphate; 1 mM KCN; 50 µM decylubiquinone; 0.8 µM antimycin, pH 7.4. The reaction was initiated by addition of NADH (100 µM). The activity of NADH CoQ oxidoreductase was measured as the rate of oxidation of NADH at 340 nm using a BioRad Model 680XR microplate reader (Hercules, CA). The activity of complex I was expressed as nmol oxidised NADH/min/mg protein. The activity of complex II was measured in buffer solution containing mitochondrial fractions (0.03 mg/ml), 100 mM potassium-phosphate, 20 mM succinate, 0.8 µM antimycin, 50 µM rotenone, 2 µM KCN, 50 µM DCIP, pH 7.4. The reaction was initiated by the addition of 50 µM decylubiquinone. The activity of succinate:DCIP oxidoreductase was measured as the rate of reduction of 2, 6-DCIP at 600 nm with 520 nm as reference wavelength. The activity of complex II was expressed in nmol reduced DCIP/min/mg protein. The activity of complex III was measured in buffer solution containing mitochondrial fractions (0.03 mg/ml), 35 mM potassium-phosphate, 5 mM MgCl₂, 2.5 mg/ml BSA, 50 mM rotenone, 1.8 mM KCN, 2 mM

decylubiquinone, pH 7.5. The reaction was initiated by the addition of 125 µM cytochrome *c*. The activity of ubiquinol:cytochrome *c* reductase was measured as the rate of reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength. The activity of complex III was expressed in nmol reduced cytochrome *c*/min/mg protein. The activity of complex IV was measured in a buffer solution containing mitochondrial fractions (0.1 mg/ml), 50 mM potassium-phosphate, pH 6.8. The reaction was started by the addition of 75 µM cytochrome *c* previously reduced with sodium borohydride and measuring the absorbance at 550 nm. The activity of cytochrome *c* oxidase was expressed as nmol oxidised cytochrome *c*/min/mg protein.

XF24 microplate-based respirometry

The Seahorse XF24 (Seahorse Biosciences, North Billerica, MA) microplate analyser simultaneously measures in real time the mitochondrial oxygen consumption rate (OCR), and the extracellular acidification rate (ECAR) as an indicator of glycolysis in the ageing rat tissue. Briefly, brain sections were immediately dissected into 200 µm slices and centred onto nylon inserts. The slices were then transferred to artificial cerebrospinal fluid (aCSF) at 23 °C containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl₂, 5 mM HEPES, 25 mM glucose and 230 µM pyruvate. The inserts were transferred facedown to the XF Islet Capture Microplates containing 675 µl of aCSF for the measurement of oxygen consumption. To control for temperature sensitive fluctuations in oxygen fluorescence emission, four wells contained blank inserts. Afterwards, tissue slices were incubated in a CO₂-free incubator at 37 °C for 1 h to equilibrate for temperature and pH. The microplate was then loaded into the XF24 and further incubated for 15 min by 3 min mix, 2 min wait cycles before commencement of the assay. The XF assay was performed as previously described (Wu et al. 2007). After determination of the basal respiration in the tissue, oligomycin (2 µM), carbonyl cyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP, 500 nM), and antimycin (3 µM) were sequentially added to the aCSF and the OCRs for each tissue sample were quantified for 2 min as previously described (Beach et al. 2010). The basal control rate (BCR) and uncoupling ratio (UCR) was used to quantify maximum mitochondrial respiration. The

BCR is a measure of the proximity of the basal level of respiration is to the maximum level of respiration (i.e., basal/maximum). The closer this ratio is to 1, the lower the maximum level of respiration, and provides a good representation of mitochondrial dysfunction. The UCR is a measure of mitochondrial functional integrity and measures the ratio of uncoupled to physiologically normal respiration levels (i.e., maximum/basal). The higher the maximum level of respiration, the greater the mitochondrial functional integrity.

Immunohistochemical fluorescence staining for 4-hydroxynonenal and poly(ADP)-ribose

Brain whole mounts were immersion-fixed with ice cold 70 % ethanol for 20 min followed by 4 % paraformaldehyde (PFA), pH 7.4 for 10 min at 4 °C, which were then used for immunohistochemical staining. Fixed brain whole mounts were permeabilised with 1 % Triton-X 100 in DPBS for 30 min at room temperature and non-specific binding was blocked with 1 % BSA in DPBS for 2 h before application of primary antibodies (Table 1). All specific primary mAb or pAb antibodies were diluted in 1 % BSA in PBS and incubated overnight at 4 °C. Brain whole mounts were washed three times with DPBS and incubated for 1 h at 37 °C with the appropriate labeled secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG coupled to Alexa 488 or Alexa 594). Nuclear staining was performed using DAPI (1 µg/ml 5 min, ambient temperature). The slides were washed three times in DPBS at ambient temperature and coverslips mounted on glass slides with Flouromount-G. The slides were examined using an Olympus BX60 fluorescence microscope fitted with a SensiCam digital camera (Kelheim, Germany). Titration series and conjugate experimental controls were performed on both primary and secondary antibodies to minimize background noise, maximize stain sensitivity and avoid non-specific staining and cross-reactivity.

CD38 knockdown via siRNA

Primary cortical rat neurons were prepared from E18 rat embryos of either sex. Embryonic brain tissues were digested with papain (1.0 mg/ml and 5 mM EDTA in HBSS, 37 °C for 20 min), washed in trituration buffer (0.1 % DNase, 1 % ovomucoid/1 % bovine serum

albumin in HBSS), and transferred to culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1 % B-27 supplement, 1 % Glutamax, 1 % antibiotic/antifungal, 0.5 % HEPES buffer, and 0.5 % glucose. Primary rat cortical neurons were transfected with 150 nM stealth siRNA directed towards CD38 (CD38si, Invitrogen) or with a negative control siRNA with similar guanosine and cytosine (GC) content as CD38si (siCon, Invitrogen). The sense sequence is 5'-UUU GGC AGU CUA CAU GUC UCA UCU C-3'. Lipofectamine (invitrogen) was used as the transfection reagent at a final concentration of 2 µl/ml. Cells were transfected with lipofectamine complexes diluted in DMEM/F-12 without serum or antibiotics. Transfection efficiency was determined to be 86 ± 3 % using an FITC-tagged siRNA and counting the number of fluorescing cells after 24 h ($n = 10$). Supplemented Neurobasal media was added to the transfection medium after 6 h. CD38. Real-time PCR and immunohistochemistry (see Table 1) was used to determine the effectiveness of CD38si knockdown after 48 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The primer sequences were as follows: CD38 forward (CTC-TGT-CTT-GGC-GTC-AGT-ATC-CTG), reverse (AGC-AAG-GTA-GCC-TAG-CAG-CGT-GTC); and GAPDH forward (AAC-AGC-GAC-ACC-CAC-TCC-TC), reverse (GG A-GGG-GAG-ATT-CAG-TGT-GGT). After an additional 24 h, NADase, cyclase, PARP, and SIRT1 activities, and intracellular NAD⁺ levels were measured as mentioned above. Extracellular lactate dehydrogenase (LDH) activity was used to measure cellular viability in CD38 knockout cells using a colorimetric assay kit (Cayman, Michigan, USA).

Bradford protein assay for the quantification of total protein

DNA damage, total antioxidant capacity, PARP activity, NAD⁺ concentration, nuclei and mitochondrial protein content, and mitochondrial function were corrected for variations in protein concentration using the Bradford protein assay described by Bradford (Bradford 1976).

Data Analysis

Results obtained are presented as the means $\pm$ the standard error of the mean (SEM) of at least eight

animals per age group analysed in duplicate. One way analysis of variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to determine statistically significant differences between treatment groups p less than 0.05 ($p < 0.05$).

Results

Age-related increase in oxidative stress in the form of protein carbonyls, lipid peroxidation and DNA damage

To confirm that oxidative stress increases with age in the rat brain, *o*- and *m*-tyrosine levels (resulting from hydroxyl radical attack on the amino acid phenylalanine) were measured in selected brain regions to assess extent of oxidative damage to protein (Davies et al. 2001; Poljak et al. 2003). Figure 2 shows the age-related changes in *o*- and *m*-tyrosine (Fig. 2a) levels in the cortex, hippocampus, cerebellum and brainstem. A significant increase in both *o*- and *m*-tyrosine formation with age was observed in all brain regions investigated, and appeared to accumulate at an accelerated rate after middle age (i.e. 12–24 months). Interestingly, the increase in protein oxidation in the cerebellum appears less pronounced compared to the other brain regions.

Peroxidative damage to cellular lipid constituents was determined using western blot analysis of the lipid peroxidation marker 4-HNE. An increase in 4-HNE levels was observed at both 12 and 24 months in the cortex ($p < 0.01$), hippocampus ($p < 0.01$), cerebellum ($p < 0.01$), and the brain stem ($p < 0.01$) (Fig. 2b, c). To further validate these results using immunohistochemical fluorescence staining, we stained frozen sections of the selected brain regions for 4-HNE (Fig. 2d). Two age related changes were clearly visible: (1) there was an increase in 4-HNE staining with age in all brain regions consistent with results reported previously using western blot analysis (Fig. 2b, c) and (2) aged tissue appeared to demonstrate significantly lower cell volume, indicative of extensive cell loss occurring during the ageing process (Fig. 2d).

The ageing process is known to affect DNA repair leading to increased vulnerability to DNA damage (Bohr 2002). We assessed DNA damage by measuring the phosphorylation of H2AX at Serine139 using a

fluorometric assay. Increased DNA damage was observed in all aged brain regions by 12 months of age compared to 3-month old rats (Fig. 2e) ($p < 0.01$). With the exception of the cerebellum, the greatest increase in DNA damage occurred between 12 and 24 months.

To assess the function of endogenous antioxidant systems with age, we measured total antioxidant capacity (TAC) *in situ* using the Trolox (tocopherol) assay. A significant reduction in total antioxidant activity was observed in the hippocampus and cortex by 12 months of age, and in all brain regions by 24 months (Fig. 2f). A more rapid rate of decline in TAC appeared to occur after middle age (i.e. 12–24 months) in all brain regions, consistent with increased oxidative stress with ageing.

Impaired mitochondrial respiratory chain activity with ageing

Although mitochondrial production of ROS is known to increase with advancing age (Rebrin and Sohal 2004), changes in the activity of the mitochondrial respiratory chain complexes in parallel with ATP production and cellular redox status during ageing in the brain has not been previously reported. We measured the activities of the mitochondrial respiratory complexes as shown in Fig. 3. The activity of the complexes were significantly decreased by 12 months of age in all brain regions with the greatest drop in activity again occurring after middle age (i.e. 12–24 months) (Fig. 3a–d).

We also investigated whether the observed deficiency in respiratory chain function was related to cellular oxygen consumption, by performing a bioenergetics assay using Seahorse XF24 (Seahorse Bioscience). Our data shows a significant increase in BCR (Fig. 3e) and a decline in UCR (Fig. 3f) ratios between brain tissue obtained 3-month old rats compared to 12- and 24-month old rats respectively. We also observed a significant increase in ECAR (Fig. 3g) in the ageing rat brain.

Increased PARP expression and ADP-ribosylation in the aging rat brain

To investigate the effect of DNA damage on poly-ADP-ribosylation, by 12 months of age all brain regions investigated showed an increase in

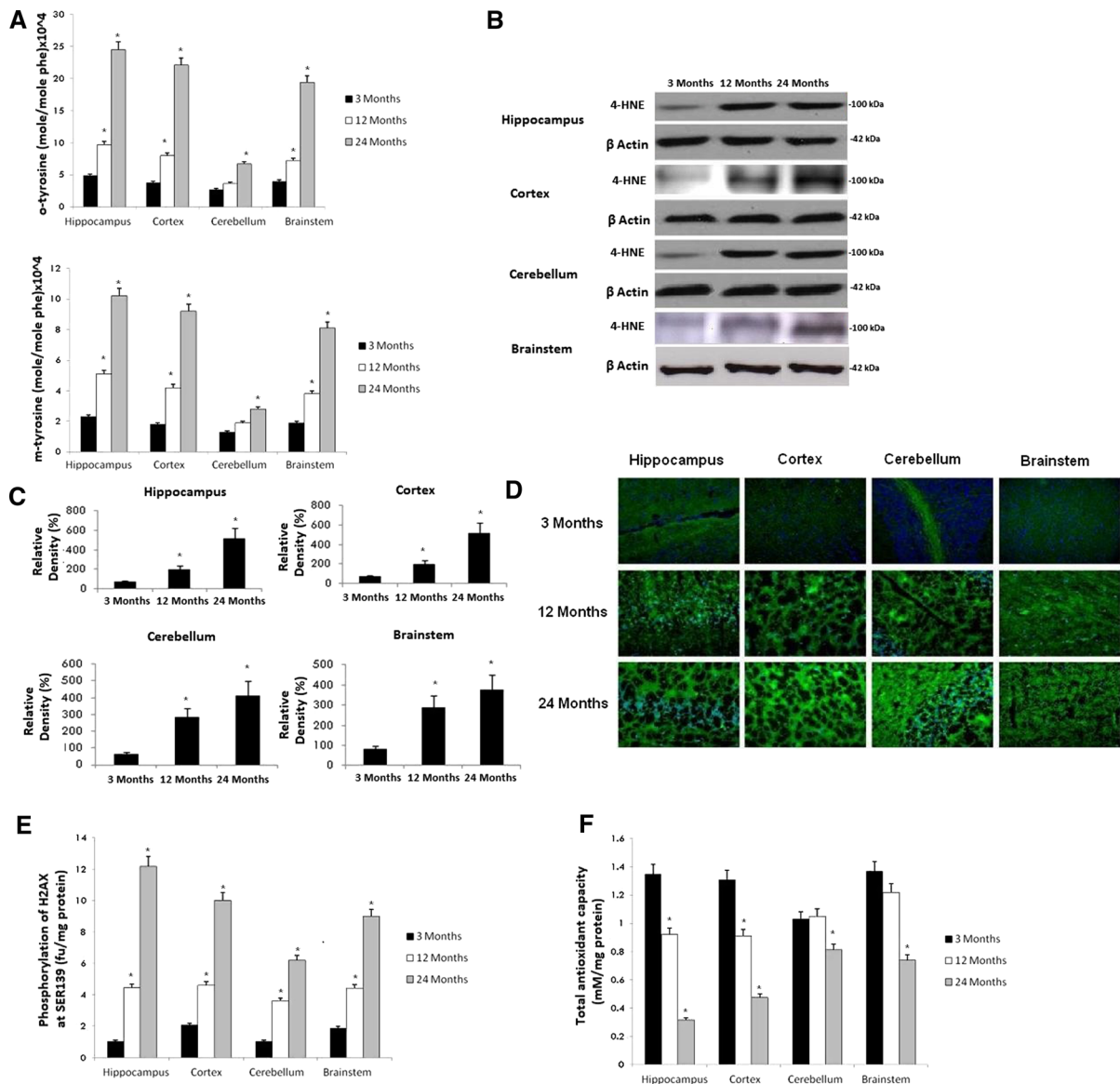


Fig. 2 Evidence for increased oxidative stress and reduced antioxidant capacity in selected brain regions with age. **a** Increased levels of *o*- and *m*-tyrosine were reported in the brain after 12 months of age compared to 3-month old rats. All values are mean $\pm$ SEM from brains from eight different rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **b** Increased oxidative damage to lipids in selected brain regions with age analysed by western blotting using anti-4HNE antibody. The blots shown are representative data from an experiment repeated eight times. **c** Graphs are mean $\pm$ SEM brains from brains of eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **d** Immunohistochemical detection of 4-HNE in the frontal lobe,

hippocampus, occipital lobe, and cerebellum from 3-, 12- and 24-month old rats. 4-HNE (green) and DAPI (blue). Higher immunoreactivity for 4-HNE was observed in 12 and 24 month old rats compared to 3-month old rats. **e** Increased DNA damage was reported in selected brain regions with age. DNA damage was measured by the fluorescence of phosphorylated H2AX at Serine139. All values are mean $\pm$ SEM from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **f** Total antioxidant capacity significantly declined in the rat brain with ageing. All values are mean $\pm$ SEM from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. (Color figure online)

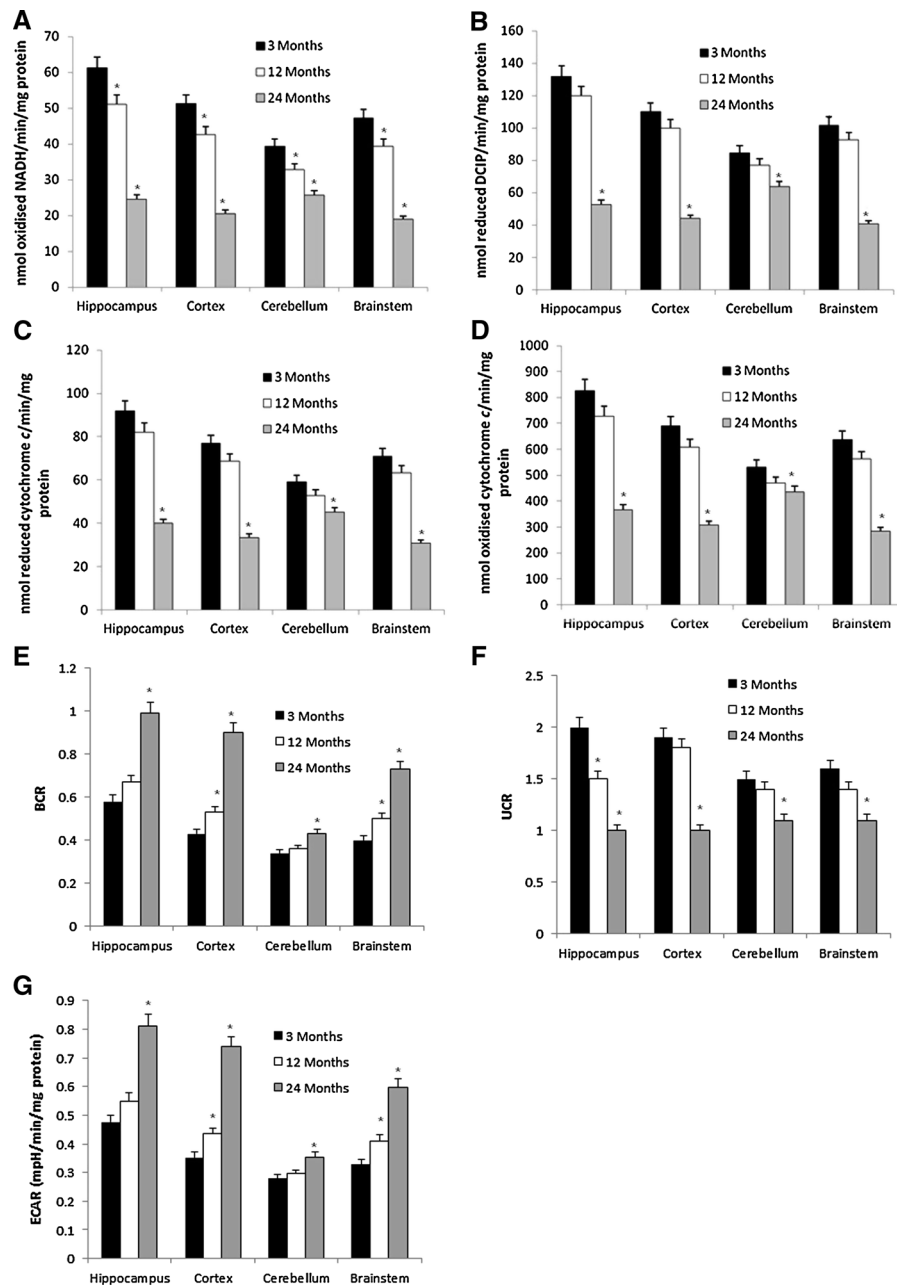


Fig. 3 Oxidative stress-mediated reduction in mitochondrial respiratory chain activity in the brain with age. Reduced **a** complex I, **b** complex II, **c** complex III, and **d** complex IV, activities at 24 months of age. All values are mean $\pm$ SEM from brains of eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **e** The basal control rate (BCR) is a measurement of how close the basal level of respiration is to the maximum level of respiration (i.e., the closer this ratio is to 1 the lower the maximum level of respiration, and thus represents an indication of mitochondrial malfunction). Significance

$*p < 0.01$ compared to 3-month old rats. **f** The uncoupling control ratio (UCR). This is a measurement of mitochondrial functional integrity and measures the ratio of uncoupled to physiologically normal respiration levels (i.e., the larger this value the greater the maximum level of respiration, and thus, the greater the mitochondrial function). Significance $*p < 0.01$ compared to 3-month old rats. **g** The ECAR is a measure of lactate formed during glycolytic energy metabolism and represents an indicator of cellular glycolysis. Significance $*p < 0.01$ compared to 3-month old rats

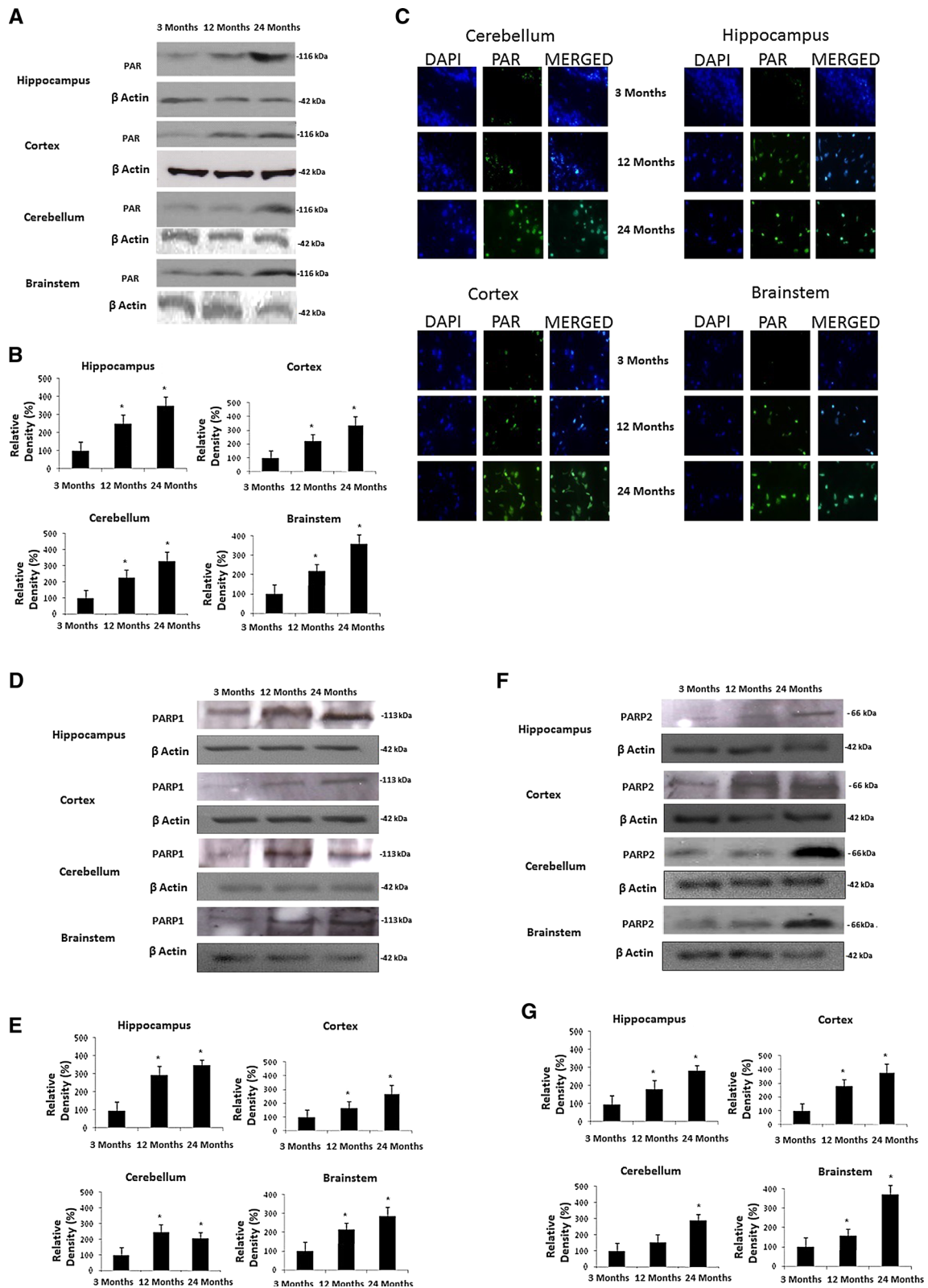


Fig. 4 Increased poly(ADP-ribose) activity in the brain with age **a** Immunoblotting for in situ accumulation of poly(ADP-ribose) in (i) cortex, (ii) hippocampus, (iii) cerebellum, and (iv) brainstem in the brain with ageing using anti-Poly(ADP-ribose) (10H) antibody. The blots shown are representative data from an experiment repeated eight times. **b** Graphs are mean $\pm$ SEM brains from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **c** Immunohistochemical detection for poly(ADP-ribose) in the hippocampus, cortex, cerebellum and brainstem from 3-, 12- and 24-month old rats. Poly(ADP-ribose) (green) and DAPI (blue). Higher immunoreactivity for poly(ADP-ribose) was observed in 12- and 24-month old rats compared to 3-month old rats. **d** Immunoblotting for PARP-1 expression in (i) cortex, (ii) hippocampus, (iii) cerebellum, and (iv) brainstem in the brain with ageing using anti-poly(ADP-ribose) (10H) antibody. The blots shown are representative data from an experiment repeated eight times. **e** Graphs are mean $\pm$ SEM brains from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **f** Immunoblotting for PARP-2 expression in (i) cortex, (ii) hippocampus, (iii) cerebellum, and (iv) brainstem in the brain with ageing using anti-Poly(ADP-ribose) (10H) antibody. The blots shown are representative data from an experiment repeated eight times. **g** Graphs are mean $\pm$ SEM brains from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. (Color figure online)

poly(ADP-ribose)lated protein complexes using western blot (Fig. 4a, b), and immunohistochemistry (Fig. 4c). We also measured PARP-1 and PARP-2 expression in nuclear extracts from the cortex, hippocampus, cerebellum and brain stem of ageing rats. Our results show a significant ($p < 0.05$) up-regulation of PARP-1 and PARP-2 expression in all brain regions by 12 months of age (Fig. 4d–g). Consistent with the increase in PARP-1 and PARP-2 expression.

Alteration in NAD⁺ and ATP levels with ageing

To determine the effect of ageing on brain levels of PARP substrate NAD⁺, we measured the intracellular NAD⁺ content in selected regions of the ageing rat brain. We found a reduction with age in the NAD⁺ content in the each of the brain regions analysed (Fig. 5a) with the greatest decrease occurring between 12 and 24 months. This change in NAD⁺ occurred in parallel with a significant increase in NADH (Fig. 5b) ($p < 0.01$) and a significant decline in ATP levels (Fig. 5d), and the NAD⁺:NADH ratio at 24 months (Fig. 5c) ($p < 0.01$). The decline in brain NAD⁺ content and NAD⁺:NADH ratio after 12 months of age appeared less pronounced in the cerebellum.

CD38 is a multifunctional enzyme that has been shown to catabolise NAD⁺ as its major enzymatic

activity. To determine the effect of ageing on CD38 function, we measured both the ADP ribosyl cyclase, and NADase activities of CD38 in brain tissue homogenates. Compared to young adults, NADase (Fig. 6a) ADP ribosyl cyclase (Fig. 6b) and activities both significantly increased in middle age rat brain slices compared to young adult controls. CD38 activity remained significantly higher in old age rats compared to middle age and young controls. With this respect, we focused on the possible role of CD38 not only as a secondary messenger enzyme, but also as a key regulator of NAD⁺ levels and NAD-dependent deacetylase activity in vivo.

Next, we determined the effect of NAD⁺ decline on SIRT1 deacetylase activity. SIRT1, an NAD⁺ dependent deacetylase, is primarily localised in the nucleus (Sauve et al. 2006). Therefore, we assessed the activity of SIRT1 in nuclear extracts from selected regions of the brain in aging rats. By 12 months a significant decline in SIRT1 activity in all brain regions was observed with the greatest decline again occurring between middle (12-month) and older age (24 months) (Fig. 6c).

CD38 is the major regulator of neuronal NAD⁺ levels, PARP and SIRT1 activity

To date, the mechanisms which regulate NAD⁺ levels in neuronal cells are not well established. While CD38 has been shown to be the main NADase in the rodent liver, the role of CD38 in modulating NAD⁺ levels in neuronal cells has not been demonstrated. Using CD38si RNA, we are the first to show that the ADP ribosyl cyclase (Fig. 7c), and NADase (Fig. 7d) activities of CD38 are absent in CD38 knockout neuronal cells. Our data also shows that NAD⁺ levels are fivefold higher in CD38 knockdown neuronal cells compared to control neurons expressing functional CD38 (Fig. 7e). This provides clear evidence supporting the role of CD38 in the regulation of intracellular NAD⁺ levels in the brain.

To further examine the role of CD38 on oxidative stress, we measured extracellular LDH activity as a measure of cell viability. Our results show that LDH activity is significantly reduced in CD38 knockout cells compared to controls (Fig. 7f). These findings suggest that disruption of CD38 may protect cells from oxidative damage by increasing the amount of NAD⁺ available for cellular repair processes.

As CD38 can regulate neuronal NAD⁺ levels, we investigated the role of nuclear CD38 on SIRT1

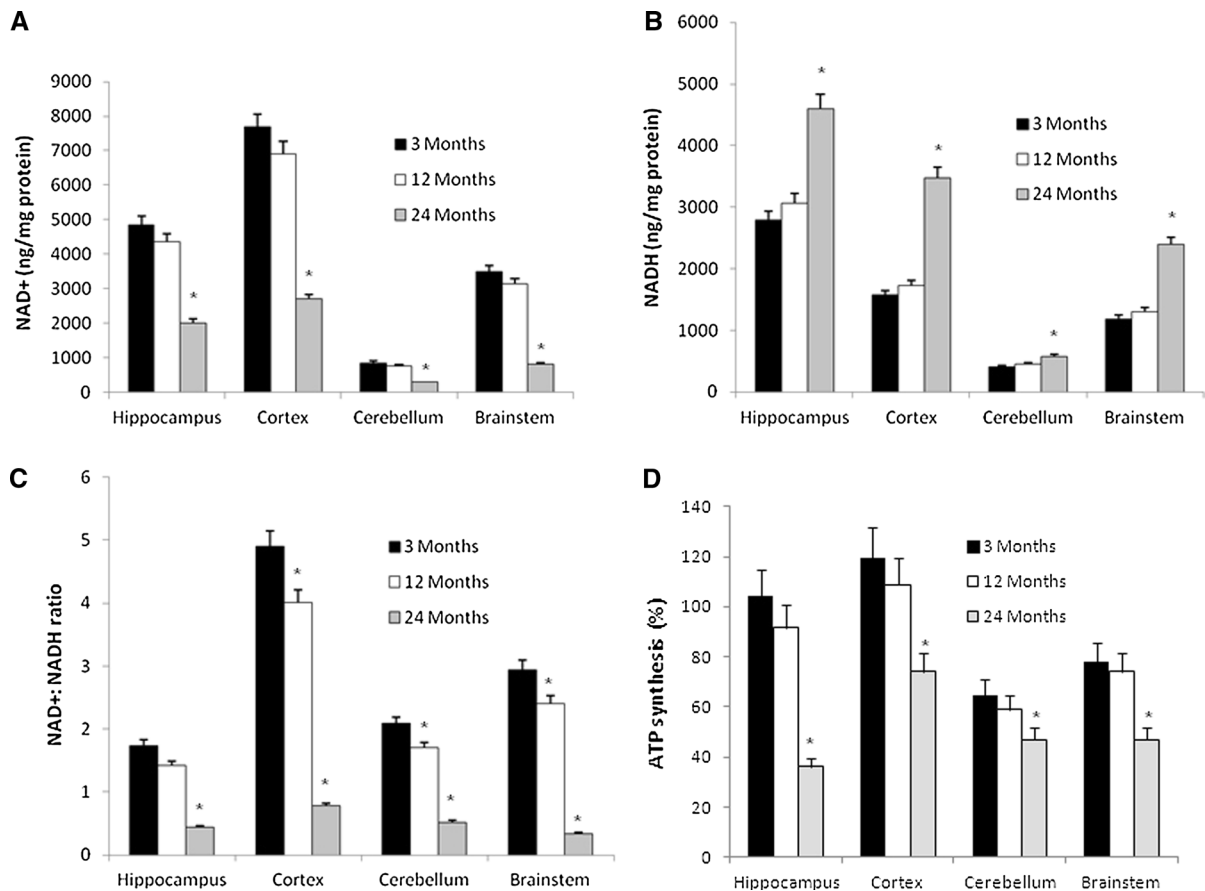


Fig. 5 Increased PARP activation alters pyridine nucleotide metabolism in the ageing brain. **a** NAD⁺ content, and **b** NADH content were determined in the ageing brain using a spectrophotometric assay. All values are mean $\pm$ SEM from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **c** NAD⁺:NADH ratio was determined as the total NAD⁺ content divided by total NADH

levels. All values are mean $\pm$ SEM from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats. **d** ATP levels were determined in the ageing brain sections using the luciferase assay. All values are mean $\pm$ SEM from brains from eight rats for each age group. Significance $*p < 0.01$ compared to 3-month old rats

activity in these cells. Our data also shows that SIRT1 activity is increased more than tenfold in CD38 knockout cells compared to neuronal cells expressing functional CD38 (Fig. 7g). This experiment confirms that CD38 can regulate nuclear PARP and SIRT1 activities. This effect is likely to be mediated by the availability of the essential substrate NAD⁺.

Discussion

In this study we report for the first time that NAD⁺ levels do in fact decrease in the brain with age, particularly after middle age, and this decrease is

associated with increased oxidative damage to proteins, lipids and DNA and increased PARP activity. This study is also the first to report on the levels of the specific markers of protein oxidative damage, *o*- and *m*-tyrosine, in ageing rat brain. Few studies have addressed the question of whether oxidative processes during ageing vary by brain region. Here, we show that oxidation of tissue lipid, protein and DNA is more apparent in the hippocampus and cortex than the brainstem and cerebellum. This is particularly relevant to the study of neuropathologies such as AD, where extensive loss of cholinergic neurons and synapses is observed in the cerebral cortex and certain subcortical regions such as the hippocampus (Mattson 2004). Our

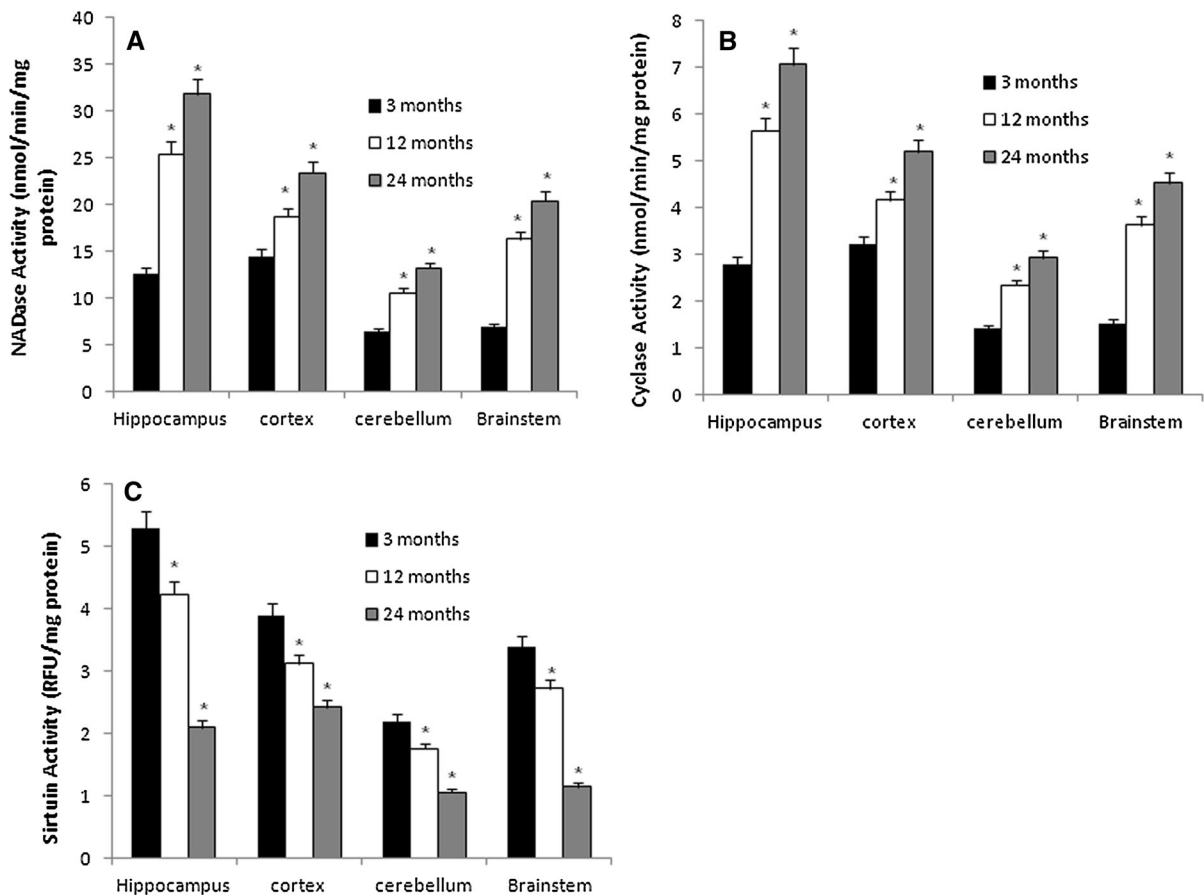


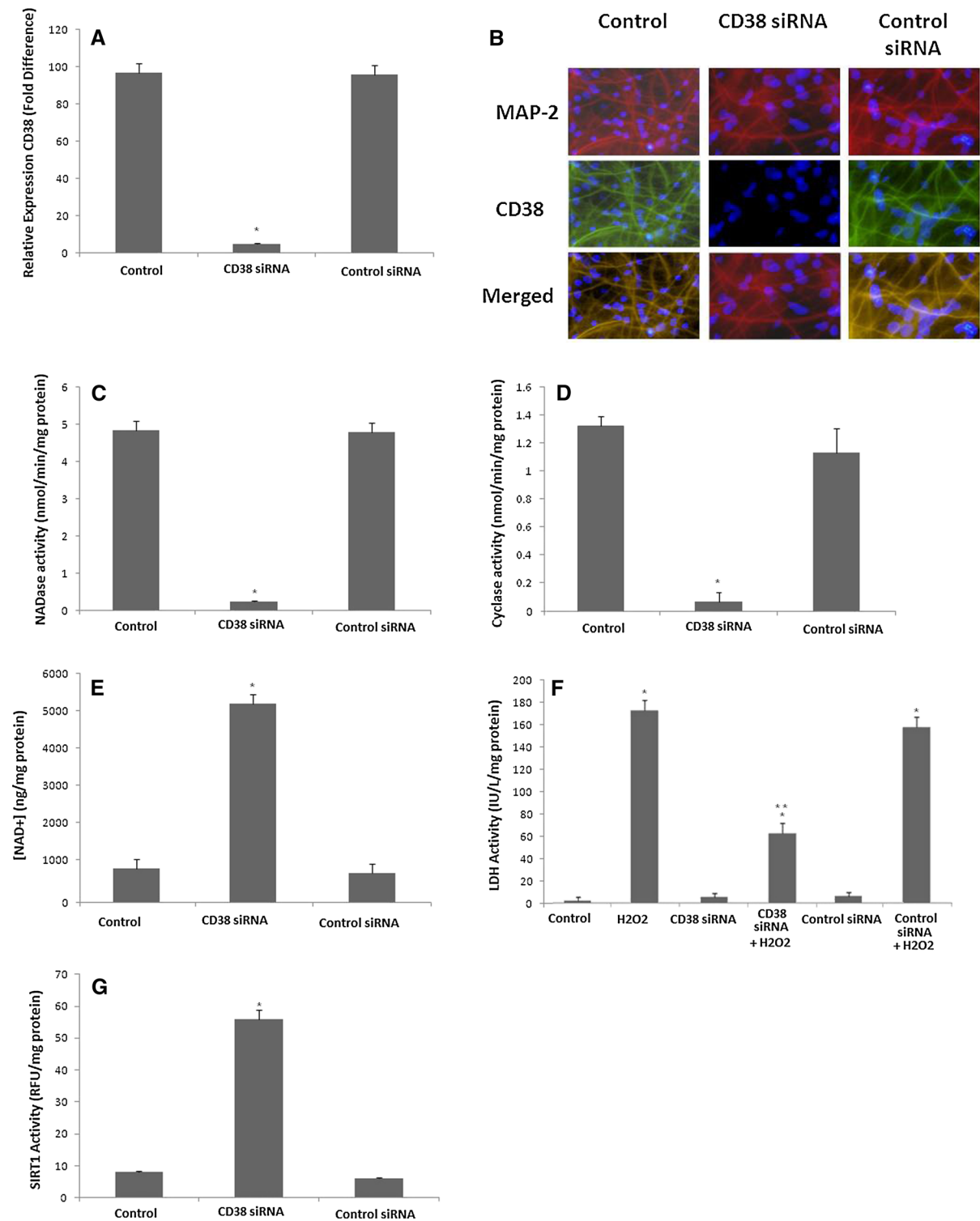
Fig. 6 Altered CD38 and SIRT1 activity in the ageing rat brain. **a** NADase and **b** cyclase activity increased significantly at 12 months of age compared to 3 month old rat brains. All values are mean $\pm$ SEM using brains from eight rats for each age group. Significance * p < 0.01 compared to 3-month old rats.

(C) Reduced SIRT1 activity was observed in the ageing rat brain after 12 months of age using a fluorimetry based assay which detected the deacetylation of lysine residues. All values are mean $\pm$ SEM using brains from eight rats for each age group. Significance * p < 0.01 compared to 3-month old rats

data show a significant increase in 4-HNE protein levels using both western blotting and immunohisto-fluorochemistry, which is consistent with a significant decline in antioxidant capacity with ageing in the brain regions under investigation. Numerous studies have shown that 4-HNE may represent a key mediator of oxidative stress induced cell death in both in vivo and in vitro studies (Kruman et al. 1997, Ahmed et al. 2002). Increased levels of 4-HNE have been shown to play an important role in the homeostasis of mitochondrial free radical production and apoptosis, leading to reduction in the efficiency of the natural antioxidant defense system and contributing to the increased toxicity reported during the ageing process (Keller and Mattson 1998, Romero et al. 1998; Zarkovic 2003). Moreover, we have previously

reported that phosphorylation of histone H2AX at Serine139 increases in the heart, lung, liver and kidney of aged female Wistar rats (Braidly et al. 2011). In the current study, increased DNA damage was observed to occur as the animal aged in all four brain regions (Fig. 2e) assessed, consistent with the increase in protein oxidation (Fig. 2a) and lipid peroxidation (Fig. 2b–c). Experimental data from other laboratories have also shown a similar increase in DNA damage, such as 8-oxoguanine (8-oxoG) in the rat brain with age (Nakae et al. 2000).

Evidence for reduced activities of mitochondrial complex I–IV in ageing animals was also observed, impacting upon both redox status and ATP production. If the age-associated increase in oxygen radicals exceeds the cell's antioxidant capacity, then



considerable reductions in complex I activity will occur. Reduced complex I activity can lead to reduced NADH oxidation and increased NADH accumulation,

as seen in older rodents (Braidley et al. 2011). This results in reduced ATP production and cellular degeneration. NAD⁺ and ATP levels may therefore

Fig. 7 CD38 is a regulator of intracellular NAD^+ levels and SIRT1 activity in primary rat cortical neurons. **a** CD38 mRNA is silenced in primary rat neurons treated with CD38si RNA. Significance $*p < 0.01$ compared to non-treated cells ($n = 3$ for each treatment groups). **b** Immunohistochemical characterisation of CD38 in CD38si RNA treated neuronal cells. Staining for CD38 in rat neurons: *Top*, double staining for MAP-2/red and DAPI/blue; *Centre*, double staining for CD38/green and DAPI/blue; *Bottom*. Merged CD38/green, MAP-2/red and DAPI/blue. CD38 NADase **c** and cyclise **d** activity are diminished in CD38si RNA treated neuronal cells. Significance $*p < 0.01$ compared to non-treated cells ($n = 3$ for each treatment groups). **e** Intracellular NAD^+ levels are fivefold higher in CD38 knockdown neuronal cells compared to control neurons expressing functional CD38. Significance $*p < 0.05$ compared to non-treated cells ($n = 3$ for each treatment groups). **f** LDH activity is significantly reduced in CD38 knockout cells compared to controls. Significance $*p < 0.05$ compared to non- H_2O_2 treated cells, $**p < 0.05$ compared to non-CD38si treated cells, ($n = 3$ for each treatment groups). **g** SIRT1 activity is increased more than tenfold in CD38 knockout cells compared to neuronal cells expressing functional CD38. Significance $*p < 0.05$ compared to non-treated cells, ($n = 3$ for each treatment groups). (Color figure online)

be effectively maintained by reducing oxidative stress induced mitochondrial suppression, thereby promoting cellular longevity. Inhibition of complex I has been demonstrated in several neurodegenerative disorders. In particular, impaired complex I activity has been observed in the substantia nigra obtained postmortem from patients who suffered from Parkinson's disease (Schapira et al. 1989), and inhibition of complex I in vivo induces Parkinsonian syndromes (Betarbet et al. 2000). Defects in mitochondrial oxidative metabolism have also been observed in AD, including reduced activity of complex I and IV in post-mortem brain, particularly in cortical areas affected with the disease (Canevari et al. 1999; Mutisya et al. 2002).

Additionally, a detailed analysis of oxygen consumption indicates that the maximum mitochondrial respiration rate is impaired with age and is suggestive of mitochondrial malfunction through disturbance of electron flow (Fig. 3e–f). The maximum respiratory rate and the ECAR are related to the flux through glycolytic pathways to generate ATP. Under physiological conditions, the rate of ATP synthesis is counter-balanced against the consumption of ATP. Our data shows an increase in extracellular flux occurring with ageing in various sections of the rat brain (Fig. 3g). This increase may be attributed to an age-related glycolytic flux as the cellular constituents of the brain attempt to

recover depleted ATP stores as in response to impaired mitochondrial electron transport as indicated by a significant reduction in the maximum mitochondrial respiratory rate (Fig. 3e–f), reduced activity of mitochondrial complexes I–IV (Fig. 3a–g), and lower concentrations of intracellular NAD^+ and ATP (Fig. 5a, d). These data support our other mitochondrial and cellular functional data in various brain sections occurring during the ageing process.

The DNA damage resulting from oxidative stress is known to activate the NAD-dependent nuclear enzyme, PARP (Burkle et al. 2004, Kim et al. 2005, Schreiber et al. 2006). PARP also appears to interact with important cell cycle regulators, such as p53, thereby assuming a dynamic role in DNA damage surveillance and regulation of cell division (Bouchard et al. 2003, Alvarez-Gonzalez et al. 2006). Several lines of evidence reported here show that PARP1 and PARP2 expression increases in the ageing rat brain, in association with decreased cellular NAD^+ levels. Work by others has implicated PARP mediated NAD^+ depletion in the pathogenesis of AD. Several studies have shown that poly(ADP-ribose) accumulates at higher concentrations in the temporal and frontal cortex in the brains of Alzheimer's patients compared to control brains (Love et al. 1999; Maldonado et al. 2007). Likewise, PARP appears to be over expressed in the AD brain and implies an excessive NAD^+ turnover in susceptible neuronal cells (Love et al. 1999; Khan et al. 2007).

CD38 is a multifunctional enzyme which utilises NAD^+ as its essential substrate to generate cADPR as an important secondary messenger. The dependency of NAD^+ for cADPR hydrolase activity has been established across diverse range of species, spanning unicellular organisms to mammalian cells. However, the effect of this hydrolase activity on NAD^+ levels has not been characterised during the ageing process. Our data are the first to show a significant increase in CD38 function (in the form of cADPR and NADase activity) in the ageing rat brain. Given that 100 molecules of NAD^+ must be hydrolysed to generate one molecular of cADPR, it is highly likely that CD38 is a major regulator of intracellular NAD^+ levels. Accordingly, we found a fivefold increase in NAD^+ levels in CD38 knockout neuronal cells compared to controls. These data support the novel concept that CD38 is a major regulator of intracellular NAD^+ levels in brain cells.

Since CD38 is involved in the degradation of neuronal NAD^+ , it is highly likely that it may also regulate the activity of important NAD-dependent pathways. The current study shows that CD38 can regulate SIRT1 activities by potentially reducing the accessibility of NAD^+ to its preferred enzymatic targets. We also show that disruption of CD38 can ameliorate the cytotoxic effect of oxidative stress in primary neuronal cells, likely through increasing the availability of intracellular NAD^+ levels required to initiate NAD-dependent cellular repair processes such as PARP. In this regard, novel CD38 inhibitors may be useful to treat several neurodegenerative disorders such as AD, upon which optimal NAD^+ anabolism remains crucial to attenuate oxidative stress insult (Escande et al. 2013). The cytoprotective effect of increased NAD^+ biosynthesis via supplementation with the NAD^+ precursor nicotinic acid has been previously demonstrated (Weidele et al. 2010).

However, inhibition of CD38 may also exhibit deleterious impact on immunological function. For instance CD38 is required for inhibiting bacterial infection and knockout of CD38 can increase the vulnerability of organisms to bacterial infection. CD38 inhibition is also likely to impact on basic neuronal function as cADPR plays a crucial role in regulating astrocyte calcium waves (Banerjee et al. 2008), and the regulation of synaptic strength between neurons (Smyth et al. 2006). CD38/cADPR also signals oxytocin release (Akther et al. 2013), which regulates many social behaviours, and inhibiting this process may induce several forms of mental impairment (Brunetti and Malavasi 2012; Lopatina et al. 2012). Moreover, niacin deficiency which has been observed in patients with pellagra often progresses to a dementia similar to schizophrenia, and this could be due to impaired cADPR formation (Ban 1975; Rudin 1981; Blankfield 2013). Despite these limitations, further understanding of the cADPR and NADase activities of CD38 may yield promising pharmacological tools to slow down age-related neurodegenerative diseases.

Given the observations in the present study, intracellular NAD^+ metabolism appears to be an important regulator of the brain ageing process. Integral to DNA repair and cell survival after oxidative stress are the linked processes of NAD^+ dependent PARP activity. Increased PARP activity due to chronic oxidative insult can induce DNA damage culminating in cell

death, since prolonged PARP activation can reduce the levels of the essential substrate NAD^+ , and a subsequent increase in the critical byproduct, nicotinamide. NAD^+ depletion and increased nicotinamide can downregulate SIRT1 deacetylase activity. Therefore, hyperactivation of PARP can modulate SIRT1 protein deacetylation via the NAD^+ /nicotinamide connection (Zhang 2003). Adequate cellular NAD^+ levels promote DNA repair, and facilitate cellular metabolism and continuing viability. Strategies aimed at maintaining cellular NAD^+ levels under conditions of increased turnover during the ageing process is particularly important in the brain which is one of the most metabolically active organs, through the promotion of oxidative phosphorylation (ATP production), DNA repair (PARP activity) and gene expression (sirtuin activity) (Braid et al. 2008, 2011, 2012).

Furthermore, while it is clear from our data that NAD^+ , and NAD^+ -dependent pathways are altered with ageing, elucidating the role of NAD^+ in brain ageing remains unclear. Specifically, the effect of chronic NAD^+ depletion on changes in gene expression or allosteric regulation of dependent enzymes has not been previously investigated. Optimal neuronal activity, synaptic plasticity and cognitive function are associated with changes in gene expression related to important physiological processes, such as lipid metabolism, inflammation and oxidative stress, endocrine pathways, and insulin signalling (Landfield et al. 2007; Blalock et al. 2010; Blalock et al. 2011; Latimer et al. 2011; Craft et al. 2012; Chen et al. 2013; Thibault 2013). The association of these alterations in gene expression to neuronal activity or plasticity, and NAD^+ decline has not been investigated. The key questions are to determine whether: (1) genes that code for NAD^+ -dependent pathways have a greater probability of changing with ageing compared to non- NAD^+ related enzymes; and (2) should any change in the genetic expression of NAD^+ -dependent enzymes shift in the same direction as NAD^+ and ageing? Answers to these unsolved questions may help to provide additional support for this complex “physiologically” aged experimental model, and provide additional data on NAD^+ -interactions with brain ageing.

In summary, altered NAD^+ metabolism in the ageing brain provides the hypothesis for neurobiological mechanisms mediating the behavioural, cognitive and neurophysiological changes associated with

the ageing process. Our work identifies the CD38 as a novel and potentially effective therapeutic target for oxidative stress mediated CNS disorders.

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