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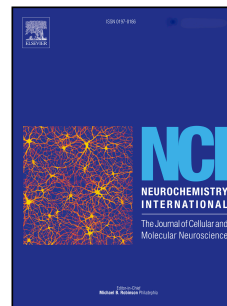

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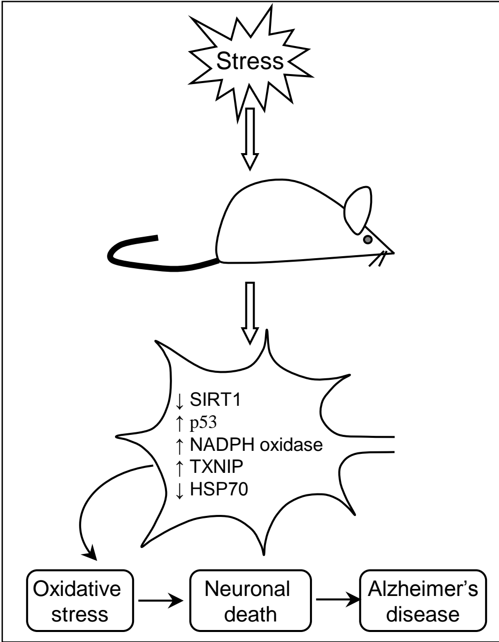
Stress
↓ SIRT1
↑ p53
↑ NADPH oxidase
↑ TXNIP
↓ HSP70
Oxidative stress
Neuronal death
Alzheimer's disease

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Chronic stress alters the expression levels of longevity-related genes in the rat hippocampus

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Abbreviations: AD, Alzheimer's disease; BDNF, brain derived neurotrophic factor; GCR, glucocorticoid receptor; HDAC, histone deacetylases; HPA, hypothalamic

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pituitary adrenal; HSF1, heat shock transcription factor 1; HSP, heat shock proteins; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; NAD, nicotinamide-adenine dinucleotide; ROS, reactive oxygen species; RU486, mifepristone; SIRT1, Silent information regulator two; TNF, tumour necrosis factor; TXNIP, thioredoxin-interacting protein.

ABSTRACT

The molecular mechanisms underlying the negative effects of psychological stress on cellular stress during aging and neurodegenerative diseases are poorly understood. The main objective of this study was to test the effect of chronic psychological stress, and the consequent increase of circulating glucocorticoids, on several hippocampal genes involved in longevity. Sirtuin-1, p53, thioredoxin-interacting protein, and heat shock protein 70 were studied at the mRNA and protein levels in stressed and non-stressed animals. Stress treatment for 10 days decreased sirtuin-1 and heat shock protein 70 levels, but increased levels of p53, thioredoxin-interacting protein and the NADPH oxidase enzyme. Examination of protein expression following two months of stress treatment indicated that sirtuin-1 remained depressed. In contrast, an increase was observed for thioredoxin-interacting protein, heat shock protein 70, p53 and the NADPH oxidase enzyme. The effect of stress was reversed by mifepristone, a glucocorticoid receptor antagonist. These data suggest that chronic stress could contribute to aging in the hippocampus.

Keywords: Chronic stress, glucocorticoids, hippocampus, oxidative stress, RU486, sirtuins.

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1. Introduction

Increased life expectancy in humans is leading to a growing interest in the study of cellular aging. Brain aging is an enormously complex process that affects multiple systems, cell types, and cellular pathways (Zeier et al., 2011). The hippocampus is one of the most studied CNS structures due to its central role in neurodegenerative disorders such as Alzheimer's disease (AD). The hippocampus is particularly sensitive to aging, as shown by age dependent impairments in synaptic plasticity and memory (Foster, 1999, 2007; Zeier et al., 2011). The hippocampus suffers from age dependent shrinking, although shrinking is not necessarily accompanied by neuronal loss (for review see Miller and O´Callagham, 2005). Furthermore, changes in several neurochemical and neurophysiological aspects of the hippocampus likely compromise its function thereby driving age dependent cognitive decline (Miller and O´Callagham, 2005).

Many age-related diseases and syndromes such as cancer, ischemia, stroke and neurodegenerative diseases such as AD and Parkinson´s disease (PD) are characterized by an altered pattern of gene expression. Changes in gene expression are due to repression or induction of key transcriptional factors that regulate crucial homeostatic cellular functions such as: inflammation, neuronal transmission, cell cycle progression, apoptosis, and DNA repair (Santa-Cruz et al., 2010). Gene expression profiles of brain tissue over the lifespan show aging-associated changes in cell growth and structural organization, inflammation, Ca^{2+} regulation and oxidative stress (Prolla, 2002; Terao et al., 2002; Blalock et al., 2003; Erraji-Benchekroun et al., 2005; Aenlle et al., 2009; Zeier et al., 2011).

Recently manipulation of metabolism and the resistance to oxidative stress have been proposed as strategies for promoting healthier aging as interventions into these systems

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promote longevity in small organisms and mammals alike (Knight, 2000; Vijg and Suh, 2005). The cellular stress response is triggered in response to stressful conditions; it promotes the activation of survival pathways that produce molecules with antioxidant and anti- and pro-apoptotic activities. There is a group of genes related to the preservation of cellular homeostasis during stressful conditions that are involved in a wide spectrum of cellular defence processes (Calabrese et al., 2010, 2011, 2012; Radak et al., 2013). These genes control a complex network of processes to ensure longevity and maintain optimum health in the long-term (Calabrese et al., 2012). This cohort of genes codes for proteins such as the sirtuins, heat shock proteins (HSP), and proteins involved in the thioredoxin system.

Sirtuins are a superfamily of class III histone deacetylases (HDAC) that are evolutionarily highly conserved (Shoba et al., 2009). Studies have shown that across various model organisms including *S. cerevisiae*, *D. melanogaster, C. elegan* and mammalian models, sirtuins have a positive effect on life expectancy (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Howitz et al., 2003; Rogina and Helfand, 2004; Santa-Cruz et al., 2010, Mercken et al., 2014). The best known member of this class of enzymes is the silent mating type information regulation 2 homolog (sirtuin) 1 or SIRT1. This enzyme plays a critical role in tumour initiation and progression by blocking senescence and apoptosis, thereby promoting cell growth and angiogenesis (Santa-Cruz et al., 2010). It has been suggested that SIRT1 protects cells from damage induced by H_2O_2, UV radiation, noxious chemicals and high caloric intake (Baur et al., 2006; Alcendor et al., 2007) through the down regulation of oxidative stress pathways (Smith, 2002; Wang et al., 2007). Thus, SIRT-1 might be a promising target for potential anti-aging interventions.

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Psychological stress, which can be defined as any disruption of homeostasis, is known to increase the rate of ageing. A 'stressor' can be any one of the myriad of internal or external challenges that cause disruption of homeostatic processes within the organism (McEwen, 1998; Miller and O´Callaghan, 2002, 2005; Smith, 2003; Lupien et al., 2005). On the level of the central nervous system, the stress response involves the activation of the hypothalamic pituitary adrenal (HPA) axis, which ultimately promotes the restoration of homeostasis. Although the HPA axis is beneficial in its acute phase, severe or repeated stressor exposure can cause adverse effects on neuronal function. Neurons in the structures involved in the stress response, such as the hypothalamus, the prefrontal cortex (PFC) and the hippocampus are particularly vulnerable to prolonged HPA axis activation (de Vasconcellos et al., 2006). It is known that chronic stress increases lipoperoxidation in the hypothalamus, PFC, and hippocampus. Within the hippocampus chronic stress also causes a decrease in cellular proliferation while concomitantly enhancing apoptotic death of hippocampal neurons (de Vasconcellos et al., 2006). Stress induces loss of hippocampal dendritic spines, inhibits neurogenesis, and hinders neuronal survival following damage (Sapolsky, 2000). In addition, stress increases the levels of pro-inflammatory markers such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α in the hippocampus (Tagliari et al., 2011; You et al., 2011), alters the expression of mitogen-activated protein kinases (MAPKs) (Meller et al., 2003; Li et al., 2009), and decreases the production of trophic factors such as the brain derived neurotrophic factor (BDNF) (Kubera et al., 2011). Finally, chronic stress also impacts microglia, the resident macrophages of the CNS, causing them to become more sensitive to inflammatory stimuli (de Pablos et al., 2006; Espinosa-Oliva et al., 2011).

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Psychological stress is a ubiquitous aspect of life, making it an important target for gaining a better understanding of the role of psychological stress and the cellular stress response in brain aging. The aim of this study was to determine whether chronic stress alters the expression of SIRT1 and other related genes in the hippocampus.

2. Materials and methods

2.1. Animals

Male albino Wistar rats (200-300 g, corresponding to an age of 2-3 months) were used for these studies. The rats were kept at a constant temperature of 22 ± 1 ºC and relative humidity (60%) with a 12-h light-dark cycle with food and water *ad libitum*.

Experiments were carried out in accordance with the Guidelines of the European Union Directive (2010/63/EU) and Spanish regulations (BOE 34/11370-421, 2013) for the use of laboratory animals; the study was approved by the Scientific Committee of the University of Seville and all efforts are made to minimize pain and discomfort and to reduce the number of animals used.

2.2. Stress model

Chronic variate stress was adapted from previously published models (Willner et al., 1987; Konarska et al., 1990; Papp et al., 1991; Murua and Molina, 1992; Muscat et al., 1992; Gamaro et al., 2003) with some modifications. Animals were divided into stressed and non-stressed control groups. Control animals were kept undisturbed in their home cages during the 10 days of treatment. A 9-day variate-stressor paradigm was used for the animals in the stressed groups. Individual stressors and length of time applied each day are listed in Table 1. The stressed/RU486 group was stressed for 9 days and received a daily dose of RU486 1 h before the 9 days of stress exposure. The following stressors were used: (a) 24 h of food deprivation; (b) 24 h of water

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deprivation; (c) 1-3 h of restraint stress, as described later; (d) 1.5-2 h of restraint stress at 4 ºC; (e) forced swimming during 10 min, as described later; (f) and 24h of social isolation. Application of stress started at different times every day, in order to minimize its predictability. Restraint stress involved placing the animal in a 21 cm x 6 cm plastic tube and adjusting it with plaster tape on the outer portion, so that the animal was unable to move; there was a 6 cm hole at the far end for breathing. Restraint at 4ºC was performed in a cold chamber. Forced swimming was carried out by placing the animal in a glass tank measuring 44 x 33 x 30 cm with 22 cm of water depth at 23 ± 2 ºC. Within the control and stressed groups, some animals were treated with RU486 (mifepristone, a glucocorticoid receptor antagonist; Sigma, St Louis, MO, USA), receiving a daily dose of 20 mg/kg in 20% DMSO in saline solution). This dosage has been previously reported (Kim et al., 2004; de Pablos et al., 2006; Mailliet et al., 2008; Espinosa-Oliva et al., 2011; Sharrett-Field et al., 2013).

For the group of animals stressed during two months we included a new set of stressors in order to avoid the adaptation of the animals. These stressors include horizontal shaker, inclination of the home cages, noise, anesthesia and isolation (Table 2).

Finally, in order to study if the effect of chronic stress is transient or permanent we included another group of animals stressed for 10 days and euthanized 14 days after the end of the last stressor.

2.3. Measurement of serum corticosterone

Rats were anesthetized with chloral hydrate (400 mg/kg) and blood was collected from the heart (n=4). The same rats were used for all the time points. Serum corticosterone concentration was measured by using an Enzyme Immunoassay (ELISA) kit following

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the manufacturer instructions (Assay Designs Correlate-EIA, Assay Designs Inc., Ann Arbor, Michigan, USA).

2.4. Retrotranscription and real-time PCR.

The hippocampus was dissected from each rat after completing the stress paradigm, snap frozen in liquid nitrogen and stored at –80ºC. Total RNA was extracted from the different treatment conditions using RNeasy® kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using QuantiTect® reverse transcription kit (Qiagen) in 20 μl reaction volume as described by the manufacturer. Real-time PCR (RT-PCR) was performed with iQ™SYBR® Green Supermix (Bio-Rad), 0.4 μM primers and 1 μl cDNA. Controls were carried out without cDNA. Amplification was run in a Mastercycler® ep realplex (Eppendorf) thermal cycler at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s, followed by a final elongation step at 72ºC for 7 min. Following amplification, a melting curve analysis was performed by heating the reactions from 65 to 95 °C in 1 °C intervals while monitoring fluorescence. Analysis confirmed a single PCR product at the predicted melting temperature. β-actin served as a reference gene and was used for sample normalization. The primer sequences are shown in table 3. The cycle at which each sample crossed a fluorescence threshold, Ct, was determined, and the triplicate values for each cDNA were averaged. Analyses of RT-PCR was done using a comparative Ct method integrated in the Bio-Rad System Software.

2.5. Spectrophotometric DNPH assay for carbonyl content determination.

Hippocampal samples were treated with 24% sodium dodecyl sulphate and boiled for 5 min. A solution of 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) in 10% trifluoroacetic acid was added to the samples (1:1 v/v). Proteins were precipitated with cold trichloroacetic acid (15%, final concentration). Protein pellets were washed three

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times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove any free 2,4-DNPH. Samples were resuspended in 6M guanidine hydrochloride in 50% formic acid overnight at room temperature. Carbonyl content was determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

2.6. Determination of hydroperoxides using the FOX reagent.

The protocol for lipid peroxidation measurement was adapted for a microplate reader (Jiang et al., 1991). 10 µl of sample was incubated with 90 µl of H_2SO_4 for 30 min. After addition of 100 µl of FOX reagent (0.5 mM ferrous ammonium sulfate, 0.2 mM xylenol orange, and 200 mM sorbitol in 25 mM H_2SO_4) the mixture was incubated at room temperature for 45 min, protected from light. The formation of ferric ions was detected by measuring the resulting colored complex with xylenol orange at 540 nm. The results were normalized to the protein content of the samples

2.7. SDS-PAGE and Western blotting

The hippocampal samples were lysed in: 15 mM Tris-HCL, pH 7,5, 150 mM NaCl, 1mM EDTA, 1 mM EGTA and 1 mM PMSF, a protease inhibitor (all from Sigma). The homogenate was centrifuged at 12000g for 20 min at 4 ºC. Protein content of the samples was estimated by the microLowry method using BSA standards (Fryer et al., 1986); 25-50 µg of protein was loaded in each lane. Protein samples were separated by SDS-PAGE (10 %), and transferred to a nitrocellulose membrane (Hybond-C extra; Amershan Life Science). Membranes were blocked with blocking buffer (5% milk in Tris-Buffer Saline: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.05 % Tween-20) for 1 h at room temperature. Membranes were then incubated using the following antibodies: anti-HSP70, anti-SIRT1 and anti-gp91^{phox} (all 1:1000), anti-p53, and anti-TXNIP (both 1:500) overnight at 5ºC in blocking buffer: 5% milk in Tris-Buffer Saline.

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β-actin antibody (1:2000) was used as a loading control. Anti-HSP70 and anti-TXNIP from Santa Cruz Biotech., Santa Cruz, CA, USA; anti-SIRT1 from Cell Signaling Tech., Lake Placid, NY, USA; anti-p53 from Calbiochem, Merck KGaA, Darmstadt, Germany; anti-gp91^{phox} from BD Biosiences (San Jose, CA); and anti-β-actin from Sigma-Aldrich. After incubation with the primary antibodies, all membranes were washed in TBST, incubated with peroxidase-conjugated anti-immunoglobulin secondary antibodies (DAKO, Produktionsvej, DK) at a dilution of 1:3000 for 1 h at room temperature in TBST. Proteins were visualized using western blotting chemiluminescence luminal reagent (Santa Cruz Biotech., Santa Cruz, CA, USA). The products were analyzed by densitometry using the Multi Gauge software (Fujifilm). The bands were normalized to actin and then expressed as percent of controls.

2.8. Statistical analysis.

Results are expressed as mean ± SD. Means were compared by a two-tailed Student's *t* test (Western blot analysis, and carbonyl and FOX measures), One-Way ANOVA (corticosterone levels in serum) or Two-Way ANOVA (body weight gain, adrenal weight and RT-PCR measures) followed by the Fischer's LSD post-hoc test. After the Two-Way ANOVA, an additional One-Way ANOVA analysis was made to find statistically significant differences between pairs of experimental groups. Alpha= 0.05. The Statgraphic Plus 3.0 software was used.

3. Results

3.1. Changes in body weight gain, adrenal gland weight and serum corticosterone.

Body weight and adrenal weight alterations are typical effects of stress (Gamaro et al., 2003). The Two-Way ANOVA analysis of factors showed that both stress ($F(1,16) = 65.95$, $p = 0.0000$) and RU486 ($F(1,16) = 36.54$, $p = 0.0000$) had an statistically

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significant effect on bodyweight gain. The interaction between the two factors was not significant ($F(1,16) = 3.42$, $p = 0.0873$ (see Table 4). Chronic stress stunted bodyweight gain (Fig. 1A); whereas non-stressed animals gained, on average, 38 g through the 10 days of the experiment, stressed rats only gained around 23 g after 10 days of chronic stress treatment. This difference in weight gain ($p < 0.01$) was even more remarkable after two months of chronic stress treatment, with a difference of 45 g of body weight gain between non-stressed and stressed animals ($p < 0.01$; not shown). The effect of 10 days of stress was reinforced by the treatment with RU486 (5 g of body weight gain; $p < 0.01$).

The Two-Way ANOVA analysis of factors showed that stress ($F(1,14) = 14.03$, $p = 0.0032$) but neither RU486 ($F(1,14) = 0.68$, $p = 0.4272$) nor the interaction between these two factors ($F(1,14) = 0.27$, $p = 0.6125$) had an statistically significant effect on the adrenal glands weight (see Table 4). After 10 days of chronic stress treatment, the adrenal glands weight increased by 32.3% ($p < 0.05$; Fig. 1B). RU486 did not alter this stress-induced increase in non-stressed and stressed animals. Metabolic changes in stressed animals differ depending on the duration, the variation, and the intensity of the stressor applied (Teague et al., 2007). In this study, the adrenal weight was not statistically different from control values after 2 months of chronic stress (not shown); these data agree with previous experiments (Rostamkhani et al., 2012).

Different types of stress in rats increase plasma corticosterone levels when applied acutely (Dhabhar and McEwen, 1997; Ricart-Jane et al., 2002; Rostamkhani et al., 2012), whereas chronic stress decreases plasma corticosterone concentration (Armario et al., 1984; Thiagarajan et al., 1989; Spencer and McEwen, 1990; Rostamkhani et al., 2012). In our experimental conditions, chronic stress induced an increase of serum corticosterone levels after 1, 3, 6 and 10 days, peaking at day 6 (Fig. 1C; $p < 0.01$)).

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However, after 2 months of treatment, chronic stress did not affect the corticosterone levels significantly.

3.2. Expression levels of SIRT1, HSP70, p53 and TXNIP.

The expression levels of SIRT1, HSP70, p53 and the thioredoxin-interacting protein (TXNIP) were measured at the mRNA and protein levels under chronic stress conditions. The Two-Way ANOVA analysis of factors showed that neither stress (F(1,15) = 0.72, p = 0.4130) nor RU486 (F(1,15) = 2.32, p = 0.1540) had an statistically significant effect on the expression of SIRT1 mRNA on their own, although there was a significant interaction between them (F(1,15) = 13.81, p = 0.0029; see Table 4) that explains the differences found with the One-Way ANOVA. So, RT-PCR analysis showed that SIRT1 mRNA was reduced to 49.4% of control levels after 10 days of chronic stress treatment (Fig. 2A; $p < 0.05$). Blockade of the glucocorticoid receptor (GCR) with RU486 in non-stressed-animals did not alter the expression levels of SIRT1, whereas treatment with RU486 in stressed-animals returned the expression levels of SIRT1 to control values (107.5%). Analysis of protein expression by Western blot showed that the effect of stress on SIRT1 levels is sustained after 2 months of chronic stress, with a 55.6% decrease with respect to control values (Fig. 3; $p < 0.05$).

Regarding HSP70, the Two-Way ANOVA analysis of factors showed that stress (F(1,12) = 5.42, p = 0.0448) but not RU486 (F(1,12) = 0.56, p = 0.5164) had an statistically significant effect on the levels of HSP70 mRNA, although there was a significant interaction between them (F(1,12) = 11.68, p = 0.0077; see Table 4) that explains the differences found with the One-Way ANOVA. Levels of HSP70 mRNA were reduced after 10 days of chronic stress treatment (33.7 % of control values, $p < 0.05$; Fig, 2B). Blockade of the GCR with RU486 in non-stressed-animals did not alter

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the expression levels of HSP70, whereas treatment with RU486 prevented this reduction partially in stressed-animals (80.9% of control values, no statistical signification compared to controls). Protein level analysis by Western Blot revealed that chronic stress increased the levels of HSP70 (+48.6%; $p < 0.05$) after 2 months of chronic stress treatment (Fig. 3).

RT-PCR showed that the expression level of the tumour suppressor gene p53 was not different from control levels after 10 days of chronic stress treatment (Fig. 2C). RU486 did not significantly alter the expression of p53 in non-stressed and sttressed-animals. The Two-Way ANOVA analysis of factors showed that neither stress ($F(1,15) = 1.46$, $p = 0.2504$) nor RU486 ($F(1,15) = 4.37$, $p = 0.0585$) had an statistically significant effect on the expression of p53 mRNA. There was no interaction between these two factors ($F(1,15) = 0.97$, $p = 0.3422$; see Table 4). However, long-term analysis by Western blot revealed that the protein levels of p53 were significantly higher in stressed-animals compared with the control group (+61.7%; Fig. 3; $p < 0.01$).

The Two-Way ANOVA analysis of factors showed that RU486 ($F(1,15) = 9.90$, $p = 0.0084$) but not stress ($F(1,15) = 3.66$, $p = 0.0798$) had an statistically significant effect on the levels of TXNIP mRNA, although there was a significant interaction between them ($F(1,15) = 10.83$, $p = 0.0065$; see Table 4) that explains the differences found with the One-Way ANOVA. Chronic stress induced a 3-fold increase in the levels of TXNIP mRNA compared with control values ($p < 0.01$; Fig. 2D). Again, treatment with RU486 in stressed-animals reversed this effect, whereas in non-stressed-animals did not alter the expression levels of TXNIP. Long-term analysis of the protein level of TXNIP by Western Blot confirmed these results, showing an increase of 61.5% in stressed animals with respect to controls (Fig. 3; $p < 0.01$).

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We also measured the mRNA expression levels of SIRT1, HSP70, p53 and TXNIP in a group of animals stressed for 10 days and euthanized 14 days after the end of the last stressor. Our results showed no statistical differences compared with the control group at this time point.

3.3. Expression levels of NADPH oxidase subunits

The expression levels of the $p22^{phox}$, $p47^{phox}$ and $p91^{phox}$ subunits of the NADPH oxidase enzyme were measured under chronic stress conditions using RT-PCR (Fig. 4). After 10 days of chronic stress treatment, the expression levels of these subunits increased with respect to control animals (+98.2 for $p22^{phox}$; +93.0% for $p47^{phox}$ and +113.1 for $p91^{phox}$; $p< 0.01$ for the three parameters). Blockade of GCR with RU486 treatment reversed this effect in stressed-animals, returning the expression levels back to control values, whereas did not alter the expression levels of the different subunits of the NADPH oxidase enzyme in non-stressed-animals. NADPH oxidase protein level (seen as $p91^{phox}$ subunit) also increased after 2 months of stress (+124.6%, $p < 0.01$; Fig. 4D) compared with the control group. The Two-Way ANOVA analysis of factors showed that both stress and RU486, as well as its interaction, have an statistically significant effect on the expression level of the different subunits of the NADPH oxidase enzyme except for stress on the p22 subunit ($p22^{phox}$: $F(1,15) = 3.25$, $p = 0.0988$ for stress; $F(1,15) = 23.29$, $p = 0.0005$ for RU486; $F(1,15) = 18.02$, $p = 0.0014$ for its interaction. $p47^{phox}$: $F(1,15) = 9.04$, $p = 0.0109$ for stress; $F(1,15) = 21.44$, $p = 0.0006$ for RU486; $F(1,15) = 18.27$, $p = 0.0011$ for its interaction. $p91^{phox}$: $F(1,15) = 12.21$, $p = 0.0050$ for stress; $F(1,15) = 16.96$, $p = 0.0017$ for RU486; $F(1,15) = 10.88$, $p = 0.0071$ for its interaction; see Table 4).

3.4. Oxidative damage

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Oxidative damage to proteins and lipids was assessed by measuring carbonyl groups and lipid peroxidation (FOX assay), respectively. After 10 days of chronic stress, no differences were found in any of the two parameters compared with the control group (data not shown); however, after 2 months of stress, lipid peroxidation increased with respect to controls (+24.0%, $p < 0.05$, Fig. 5). No significant changes were found for carbonyl groups.

4. Discussion

The large and uneven spectrum of cognitive capacity in the elderly has generated many questions about how our brain ages. The lack of uniformity in rates of aging has driven research investigating how structures such as the hippocampus are modified with age, and how certain factors such as stress, may contribute to these changes (Miller and O'Callaghan, 2003; Smith, 2003; Maras and Baram, 2012). Because of the high prevalence of stress in our modern world, the mechanisms by which stress drives deleterious processes in the hippocampus have garnered interest. For instance, stress has various cellular and systemic effects on synaptic plasticity, hippocampal connectivity, neuronal survival, hippocampal neurogenesis and memory (McEwen and Milner, 2007, Sandi and Pinelo-Nava, 2007, Segal et al., 2010; de Kloet, 2012; Bartsch and Wulff, 2015). It is also known that stress affects the hippocampal LTD and LTP (for review see Kumar, 2011) and leads to altered dendritic architecture in various regions of the hippocampus (de Kloet et al., 2005). The effects of stress on the hippocampus are complex and dynamic, and are influenced by age and gender, as well as by the context and duration of the stressor (Calabrese et al., 2007; Goel and Bale, 2009; Joels et al., 2009; Zoladz and Diamond, 2009; McEwen and Gianaros, 2011; Schwabe et al., 2011).

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The main objective of this study was to demonstrate that stress and the consequent increase of circulating GCs can alter the expression levels of some longevity-related genes in the hippocampus, a brain region critical for learning and memory, and highly sensitive to aging and stress. Recent therapeutic approaches to controlling the deleterious processes associated with aging focus on metabolic manipulations and increasing the resistance to oxidative stress by inducing the expression of groups of genes including sirtuins, thioredoxin and HSP systems. We have chosen to analyze the regulation of three genes related to longevity (SIRT1, HSP70 and TXNIP) to see the effects of stress. Due to the opposite roles of these genes in regulating oxidative stress and metabolism, our hypothesis is that the circumstances that inhibit SIRT1 and HSP70 concomitantly induce TXNIP thereby causing the most damage.

Accumulating evidence highlights the importance of SIRT1-mediated epigenetic control in memory formation and neuronal plasticity through a microRNA-mediated mechanism (Gao et al., 2010; Torres et al., 2011). SIRT1 can regulate neuronal differentiation (Donmez and Outeiro, 2013; Saharan et al., 2013) and prevent neurodegeneration in mouse models of AD (Kim et al., 2007). Moreover, Tau acetylation can be reverted by SIRT1 (Julien et al., 2009). Interestingly, SIRT1 brain-specific knockout mice show impaired cognitive abilities (Michán et al., 2010). Previous studies have shown a decrease in SIRT1 protein levels with age in the hippocampus, although SIRT1 mRNA is not altered in this structure, so post-transcriptional mechanisms might be involved (Quintas et al., 2012). Furthermore, the cumulative increase in oxidative stress during age induces a decrease of the catalytic activity of SIRT1 in tissues, possibly by a direct inactivation by reactive oxygen species (ROS) (Braidy et al., 2012). Ferland and Schrader (2011) studied the post-translational modifications of histones in response to chronic stress in the hippocampus, suggesting a

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complex pattern in histone acetylation that can contribute to stress-induced pathology. They showed that chronic stress increases SIRT-1 activity with no effect on the protein expression levels. On the other hand, Chang et al. (2009) showed that total sleep deprivation reduces the protein levels of SIRT-1 in hippocampal pyramidal cell layers. Our findings show that chronic stress applied for 10 days produced a decrease in the mRNA levels of SIRT1 in the hippocampus. This effect is sustained after up to 2 months of treatment as evidenced by the analysis of SIRT1 protein levels by Western blot. Our results do not agree with those of Ferland and Schrader (2011). This discrepancy could be due to the different stressor used, the time of exposure to the stress paradigm and the method used for measuring SIRT1 protein levels. Since the levels of SIRT1 decrease with age, our results suggest that chronic stress could accelerate aging processes in the brain through the removal of the protective action of this gene.

The neuroprotective effects of SIRT1 may be due, at least in part, to its action on p53. p53 is the first known non-histone substrate of SIRT1. Since p53 is responsible for the induction of many pro-apoptotic molecules, it plays an important role in most neurological disorders (Hong et al., 2010; Yi and Luo, 2010). P53 transcriptional activity is increased when it is acetylated in multiple lysine residues, whereas its activity is decreased when it is deacetylated. It is known that SIRT1 deacetylates p53 reducing its transcriptional activity (Luo et al., 2001; Vaziri et al., 2001). This could be one of the mechanisms by which SIRT1 is neuroprotective. In fact, it is known that deacetylated-p53 protects neurons in models of AD (Kim et al., 2007; Karuppagounder et al., 2009), amyotrophic lateral sclerosis in mice (Kim et al., 2007), and PD in mesencephalic cuts (Okawara et al., 2007). Our results show that chronic stress applied for 10 days did not significantly altered the expression levels of p53 mRNA in hippocampus. However, Western blot analysis revealed that levels of p53 protein were increased after to 2

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months of chronic stress treatment. Spange et al. (2009) suggested that acetylation of p53 is linked to its stability, preventing ubiquitination and hampering signaling for proteolitic degradation. Thus, in our experimental conditions, the decreased levels of SIRT1 after chronic stress could decrease polyubiquitination of p53, increasing its stability and leading to an increase in its protein levels. This could explain some previous studies which have shown that stress can induce apoptosis in hippocampal layers CA1 and CA3 (Zhao et al., 2007; Zhu et al., 2008; Li et al., 2010).

SIRT1 inhibition of p53 alters redox balance, through reduction in the pro-apoptotic production of ROS (Polyac et al., 1997; Hussain et al., 2004; Radak et al., 2013). Thus, Vurusaner et al. (2012) suggested that cellular levels of ROS are decreased by low levels of deacetylated p53. Conversely, high levels of acetylated p53 can induce ROS-production related genes. Based off of these findings, we wanted to study some genes that could act in opposition to SIRT1, thereby driving oxidative stress. The gene encoding TXNIP fit this criterion, as TXNIP is a multifunctional protein that plays an important role in redox homeostasis. Increased levels of this protein are associated with increased levels of oxidative stress and a decrease in life expectancy (Mousa et al., 2009). Our findings indicate that chronic stress increases the levels of TXNIP mRNA. This increase is sustained in the hippocampus after 2 months of stress, as shown by Western blot analysis.

The increased levels of TXNIP that we observed in the hippocampus of stressed rats can be indicative of increased oxidative stress in this structure. TXNIP activates proinflammatory signals and promotes oxidative stress by inhibiting thioredoxin activity (Guarente and Picard, 2005). To investigate this relationship, we measured the levels of the enzyme NADPH oxidase, a major source of free radicals. Superoxide generation by NADPH oxidase plays an important role in several neurodegenerative disorders,

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including AD (Ansari and Scheff, 2011). TXNIP is a key intermediate bridging ROS production and pathological conditions (Abais, et al., 2014; Joshi et al., 2014). Therefore, Gokulakrishnan et al. (2009) suggested that NADPH oxidase could elevate TXNIP levels thereby promoting the oxidative stress response. Our results show that increased expression of TXNIP is associated with increased expression of several subunits of the enzyme NADPH oxidase in the hippocampus. Moreover, we found an increase in ROS production (measured by levels of lipid peroxidation) after 2 months of chronic stress. Therefore, in our experimental conditions, TXNIP could represent an intermediate candidate connecting the production of ROS with chronic stress. Italiano et al. (2012) showed that p53 is a transcription factor for the $p67^{phox}$ subunit of NADPH oxidase. Our results agree with previous work showing that GCs drive oxidative stress in the hippocampus by upregulating genes related to ROS generation (You et al., 2009). Based on these various findings and our work, a network of oxidative stress pathways begins to emerge.

The relationship between SIRT1 and NADPH oxidase remains unclear. We show here that stress alters the expression of SIRT1 and several NADPH oxidase subunits. Stress decreases SIRT1 levels while concomitantly increasing NADPH oxidase subunit levels. SIRT1 is an inhibitor of free radical-mediated oxidative injuries and it executes this function by inhibiting NADPH oxidase activation (Zarzuelo et al., 2010; Kitada et al., 2011). This is also the case in the aging vasculature, supporting the idea that SIRT1 can act as an upstream inhibitor of the $p47^{phox}$ subunit (Tang et al., 2012). Therefore, our results suggest that the decreasing levels of SIRT1 during chronic stress may be a factor in the promoting increased expression of NADPH oxidase, which in turn could trigger increased expression of TXNIP. The consequent generation of oxygen free radicals would then increase oxidative stress in the hippocampus. However, establishing a causal

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relationship between SIRT1, NADPH oxidase and TXNIP requires further investigation.

Another important mediator of cellular stress is the protein family encompassing the HSPs. HSP70 has been heavily investigated in studies of longevity. HSP70 levels generally decrease with age (Gutsmann-Conrad et al., 1999; Calderwood et al., 2009). HSP70 works as a cytosolic chaperone promoting the correct folding of proteins or their degradation by the proteasome (Kavanagh et al., 2012). During aging reduced levels of HSP70 contribute to the aging process: cells lacking HSP70 accumulate damaging protein products over time (Linton et al., 2001; Horowitz et al., 2007). Humans with stable levels of HSP70 in circulating white blood cells, and animal models with adequate HSP70 levels show resistance to the detrimental effects of aging (Jin et al., 2004; Marini et al., 2004). Overexpression of HSP70 increases longevity in *C. elegans* models of aging (Lithgow et al., 1995; Walker and Lithgow, 2003). Interestingly, caloric restriction, a well-known strategy to increase longevity, increases HSP70 levels in cell culture (Heydari et al., 1993, 1995). These various reports suggest that HSP70 and protein homeostasis are important in healthy aging and longevity. Therefore, we decided to study whether chronic stress affected the expression of HSP70 in the hippocampus. Our results showed a decrease in the expression of HSP70 mRNA after 10 days of chronic stress exposure. SIRT1 deacetylates heat shock transcription factor 1 (HSF1), thereby enhancing its binding to the HSP70 promoter, promoting transcription of HSP70 (Westerheide et al., 2009; Radak et al., 2013). In our experimental conditions, the decreased levels of SIRT1 after chronic stress treatment could be an explanation for the reduction in the levels of HSP70 mRNA. Interestingly, our Western blot analysis shows an increase in the protein levels after the 2-month stress paradigm. The increase in HSP70 is likely due to compensatory protective mechanisms within the

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cell triggered by prolonged high oxidative stress. HSP70 is a chaperone that confers resistance to stress in several tissues by facilitating tissue repair and conferring resistance to oxidative damage.

The effects of chronic stress on gene expression and protein levels could be either permanent or transient. In order to study this issue, we measured the mRNA expression levels of SIRT1, p53, TXNIP and HSP70 in a group of animals stressed for 10 days and euthanized 14 days after the end of the last stressor. In these experimental conditions, we did not find statistical differences compared with the control group. This indicates that the effects of chronic stress in the expression levels of these genes are transient and reversible, demonstrating that they are stress-specific changes. However, it should be recognized that although stress-induced changes of SIRT-1, TXNIP, p53 and HSP70 were transient, oxidative stress-induced dysfunction and damage are cumulative (Harman, 1956; Edrey and Salmon, 2014). Further studies should be performed in aged animals to better elucidate the long-term effects of chronic stress on SIRT1, TXNIP, p53 and HSP70 expression levels in the hippocampus.

Finally, to demonstrate that the effects of chronic stress are due to the release of corticosteroids, we used the glucocorticoid receptor antagonist mifepristone (RU486). Although in control animals treatment with RU486 have no effect, our results show that blocking the actions of glucocorticoids restores all the parameters studied, which indicates that glucocorticoids play a central role in the deleterious effects of stress.

5. Conclusions

Advanced age and stress share several commonalities, including oxidative stress and neuroinflammation, which could contribute to impaired hippocampal function (Espinosa-Oliva et al., 2010; Maras and Baram, 2012; Bartsch and Wulff, 2015). Our

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results show that chronic stress is capable of decreasing the expression of genes involved in cell survival, while simultaneously enhancing the expression of genes involved in aging in the hippocampus. Furthermore, our results show that stress increases enzymes capable of driving oxidative stress in the hippocampus. These results provide a more complete picture of the mechanisms by which stress could contribute to the aging process in a brain structure particularly involved in age-related diseases such as AD and other dementias. The increased life expectancy in our society means that an increasing portion of the population is susceptible to age-related diseases. Our results may help to understand the devastating effects of stress on the hippocampus, and highlight the need to reduce the effects of stress on the growing population of elderly people.

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CONFLICT OF INTEREST STATEMENT

All authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Effect of stress and RU486 on the body weight, adrenal glands weight and serum levels of corticosterone. (A) Body weight gain in stressed and non-stressed animals in combination with RU486. Results are expressed in grams and are the mean ± SD of at least four animals (n = 4-5) for each group. (B) Adrenal weight (in mg) in stressed and non-stressed animals in combination with RU486. Results are mean ± SD of at least three animals (n = 3-4) for each group. (C) Quantification of serum levels of corticosterone from stressed animals. Results are mean ± SD of three animals (n = 3) per time point (except 6 days, with n = 4). Statistical significance: panels A and B, Two-Way ANOVA followed by the LSD post hoc test for multiple range comparisons (see Table 4 for statistical analysis of the main effects and its interaction), followed by an additional One-Way ANOVA to find statistically significant differences between pairs of experimental groups. Effect of stress: ‡, $p < 0.01$ and $p < 0.05$ for panels A and B, respectively. Effect of RU486: #, $p < 0.01$. Panel C, One-way ANOVA followed by the LSD post hoc test for multiple range comparisons: *, compared with the control group (0 days); +, compared with the previous time point; $p < 0.01$. (Control: non-stressed animals; S10d: animals stressed for ten days).

Figure 2. Effect of stress and RU486 on the expression levels of SIRT1, HSP70, p53 and TXNIP mRNAs in the hippocampus. Expression of SIRT1 (A), HSP70 (B), p53 (C) and TXNIP (D) mRNAs in the hippocampus of rats from the different treatments assayed were measured by RT-PCR. Control: non-stressed animals; S10d: animals stressed for ten days. Results are mean ± SD of at least three animals (n = 3-5), and are expressed as percentage of controls. Statistical significance: Two-Way ANOVA followed by the LSD post hoc test for multiple range comparisons (see Table 4 for statistical analysis of the main effects and its interaction), followed by an additional

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One-Way ANOVA to find statistically significant differences between pairs of experimental groups. Effect of stress: ‡, $p< 0.05$ for panels A and B, and $p< 0.01$ for panel D, respectively. Effect of RU486: #, $p< 0.05$ for panels A and B, and $p< 0.01$ for panel D, respectively.

Figure 3. Effect of stress on the expression levels of SIRT1, HSP70, p53 and TXNIP proteins in the hippocampus. Proteins from the hippocampus of rats under the different treatments assayed (C: Control, non-stressed animals; S2m: animals stressed for two months) were separated by electrophoresis and transferred to nitrocellulose membranes, and stained using anti-SIRT1, anti-HSP70, anti-p53 and anti-TXNIP antibodies. Total optical density of each band was calculated. Results are mean ± SD of at least five animals, and are expressed as intensity relative to control bands. Statistical significance (Two-tailed Student's t test) compared with the control rats: *, $p < 0.05$; **, $p < 0.01$.

Figure 4. Effect of stress and RU486 on the expression levels of NADPH oxidase subunits mRNAs and the amount of $gp91^{phox}$ protein in the hippocampus. Expression levels of $p22^{phox}$ (A), $p47^{phox}$ (B) and $gp91^{phox}$ (C) mRNAs were measured by RT-PCR in the hippocampus of rats from the different treatments assayed (C: Control, non-stressed animals; S10d: animals stressed for ten days). Results are mean ± SD of at leat three (n = 3-5) independent experiments, and are expressed as percentage of controls. (D) Proteins from the hippocampus of rats under the different treatments assayed (Control: non-stressed animals; S2m: animals stressed for two months) were separated by electrophoresis and transferred to nitrocellulose membranes, and stained using anti-$gp91^{phox}$ antibody. Total optical density of each band was calculated. Results are mean ± SD of five animals (n=5) and are expressed as intensity relative to control bands. Statistical significance: panels A, B and C, Two-Way ANOVA followed by the

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LSD post hoc test for multiple range comparisons (see Table 4 for statistical analysis of the main effects and its interaction), followed by an additional One-Way ANOVA to find statistically significant differences between pairs of experimental groups. Effect of stress: ‡, $p < 0.01$. Effect of RU486: #, $p < 0.01$. Panel D, Two-tailed Student's t test; *, $p < 0.01$compared with the non-stressed rats.

Figure 5. Effect of stress on carbonyl and hydroperoxides content in the hippocampus.

The content of carbonyl groups and hydroperoxides in hippocampal samples were measured after two months of stress. Results are mean ± SD of five animals (n = 5), and are expressed as percentage of control animals. Statistical significance (Two-tailed Student's t test): *, $p < 0.01$ compared with the non-stressed rats.

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Table 1. Schedule of stressors used during the chronic variate stress treatment.

Day	Stressor	Time
1	Forced swimming	10 min
2	Restraint	3 h
3	Water deprivation	24 h
4	Restrain at 4º C	90 min
5	Isolation	24 h
6	Food deprivation	24 h
7	Water deprivation	24 h
8	Restrain at 4º C	2 h
9	Food deprivation	24 h

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Table 2. Schedule of stressing agents used during the 2 month chronic stress treatment.

Day	Stressor	Time	Day	Stressor	Time
1	Anaesthesia	Blood col.	31	Water deprivation	24 h
2	Forced swimming	10 min	32	Horizontal shaker	1 h
3	Restraint	3 h	33	Inclination of home cages	5 h
4	Water deprivation	24 h	34	Non-stressed	24 h
5	Horizontal shaker	1 h	35	Non-stressed	24 h
6	Non-stressed	24 h	36	Forced swimming	10 min
7	Non-stressed	24 h	37	Restraint at 4ºC	1.5 h
8	Forced swimming	10 min	38	Noise	10 min
9	Restraint at 4ºC	1.5 h	39	Isolation	24 h
10	Noise	10 min	40	Restraint	3 h
11	Isolation	24 h	41	Non-stressed	24 h
12	Inclination of home cages	5 h	42	Non-stressed	24 h
13	Non-stressed	24 h	43	Forced swimming	10 min
14	Non-stressed	24 h	44	Restraint	3 h
15	Forced swimming	10 min	45	Inclination of home cages	5 h
16	Water deprivation	24 h	46	Isolation	24 h
17	Horizontal shaker	1 h	47	Non-stressed	24 h
18	Restraint	3 h	48	Non-stressed	24 h
19	Inclination of home cages	5 h	49	Water deprivation	24 h
20	Non-stressed	24 h	50	Restraint	3 h
21	Non-stressed	24 h	51	Restraint at 4ºC	1.5 h
22	Restraint at 4ºC	1.5 h	52	Noise	10 min
23	Forced swimming	10 min	53	Isolation	24 h
24	Isolation	24 h	54	Forced swimming	10 min
25	Noise	10 min	55	Non-stressed	24 h
26	Restraint	3 h	56	Non-stressed	24 h
27	Non-stressed	24 h	57	Forced swimming	10 min
28	Non-stressed	24 h	58	Horizontal shaker	1 h
29	Forced swimming	10 min	59	Inclination of home cages	5 h
30	Anaesthesia	Blood col.	60	Anaesthesia	Blood col.

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Table 3. Primers for RT-PCR

mRNA	Primers
β-actin	S: 5´-TGTGATGGTGGGAATGGGTCA-3´ A:5´-TTTGATGTCACGCACGATTTCC-3´
Sirt-1	S: 5´-TCATTCCTGTGAAAGTGATGACGA-3´ A: 5´-GCCAATCATGAGGTGTTGCTG-3´
TXNIP	S: 5´-CGAGTCAAAGCCTCAGGAT-3´ A: 5´-TTCATAGCGCAAGTAGTCCAAGGT- 3´
$p22^{phox}$	S: 5´-GAATTCCGATGGGCAGATCGA-3´ A: 5´-GGAQTCCCGTCACACGACCTCA-3´
$p47^{phox}$	S: 5´-ATTTGGAGCCCTTGACAG-3´ A: 5´-GATGGTTACATACGGTTCACCTG-3´
$gp91^{phox}$	S:5´-GCACAGCCAGTAGAAGTAGATCTTT-3´ A: 5´-GCTGGGATTGGAGTCACG-3´
HSP70	S: 5´- GGGCTCTGAGGAACCGAGC-3´ A: 5´- CAGCCATTGGCGTCTCTC-3´
P53	S: 5´- AGAGAGCACTGCCCACCA-3´ A: 5´- AACATCTCGAAGCGCTCAC-3´

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Table 4. Two-Way ANOVA and Multiple Range Test for the variables and factors in Figures 1, 2 and 4.

Variables		Main effects A	Main effects B	Interactions AB
Body weight gain	*F* ratio	65.95	36.54	3.42
DF (1,16)	Sig. Level	0.0000	0.0000	0.0873
Adrenal weight	*F* ratio	14.03	0.68	0.27
DF (1,14)	Sig. Level	0.0032	0.4272	0.6125
SIRT 1	*F* ratio	0.72	2.32	13.84
DF (1,15)	Sig. Level	0.4130	0.1540	0.0029
HSP70	*F* ratio	5.42	0.46	11.68
DF (1,12)	Sig. Level	0.0448	0.5164	0.0077
P53	*F* ratio	1.46	4.37	0.97
DF (1,15)	Sig. Level	0.2504	0.0585	0.3422
TXNIP	*F* ratio	3.66	9.90	10.83
DF (1,15)	Sig. Level	0.0798	0.0084	0.0065
P22	*F ratio*	*3.25*	*23.29*	*18.02*
DF (1,15)	Sig. Level	0.0988	0.0005	0.0014
P47	*F* ratio	9.04	21.44	18.27
DF (1,15)	Sig. Level	0.0109	0.0006	0.0011
P91	*F* ratio	12.21	16.96	10.88
DF (1,15)	Sig. Level	0.0050	0.0017	0.0071

Multiple Range Test	Variables	Factor A Control/Stress	Factor B Veh./RU 486
	Body weight gain	+	+
	Adrenal weight	+	-
	SIRT 1	-	-
	HSP70	+	-
	P53	-	-
	TXNIP	-	+
	P22	-	+
	P47	+	+
	P91	+	+

Main factors: (A) stress treatment; (B) product injected i.p. Numbers in parentheses are

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degrees of freedom used for the calculations of F ratios; $\alpha = 0.05$. Multiple Range Test (Least Squared Differences, LSD): +, significant; -, non significant.

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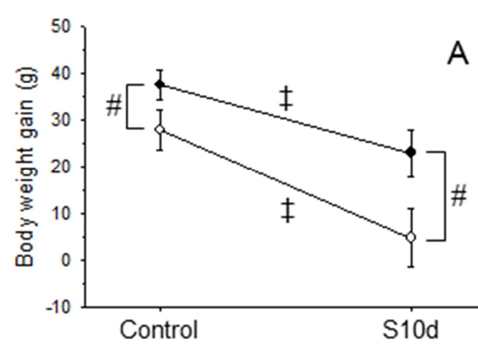
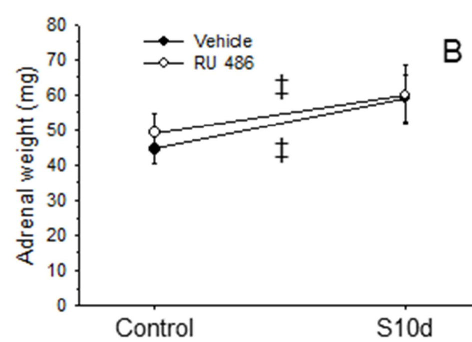
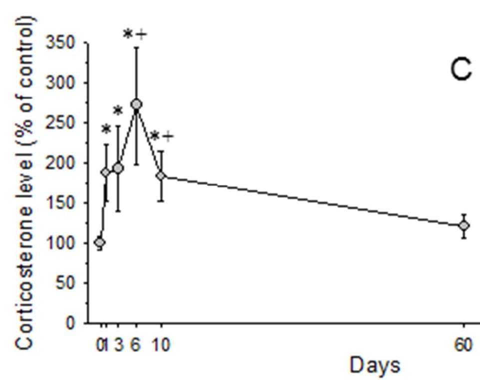
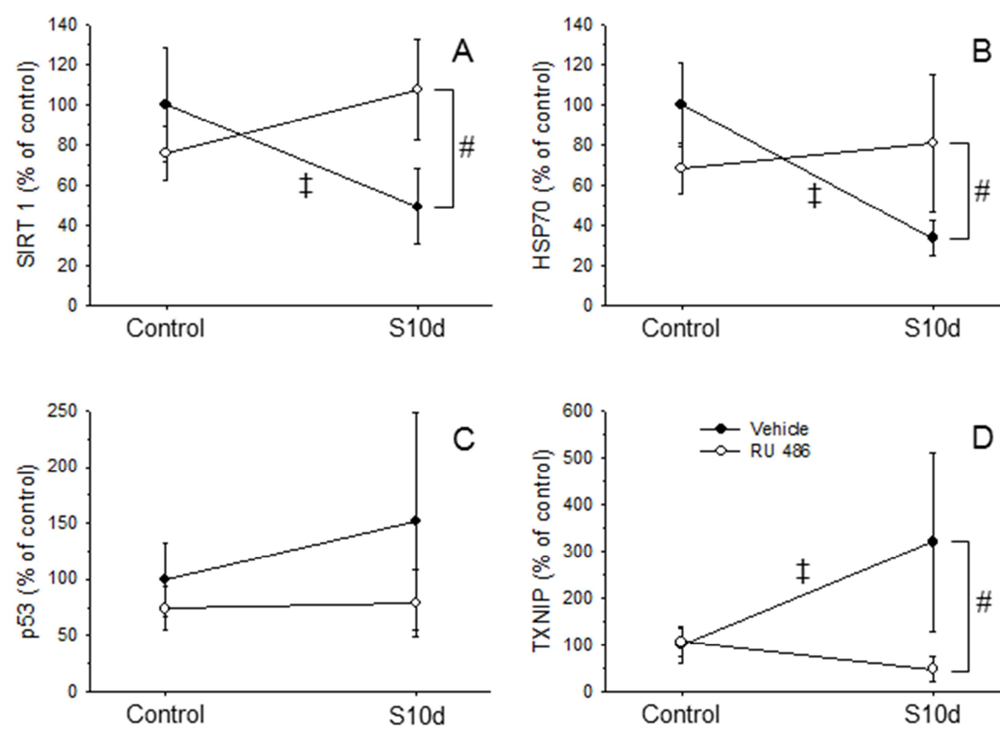
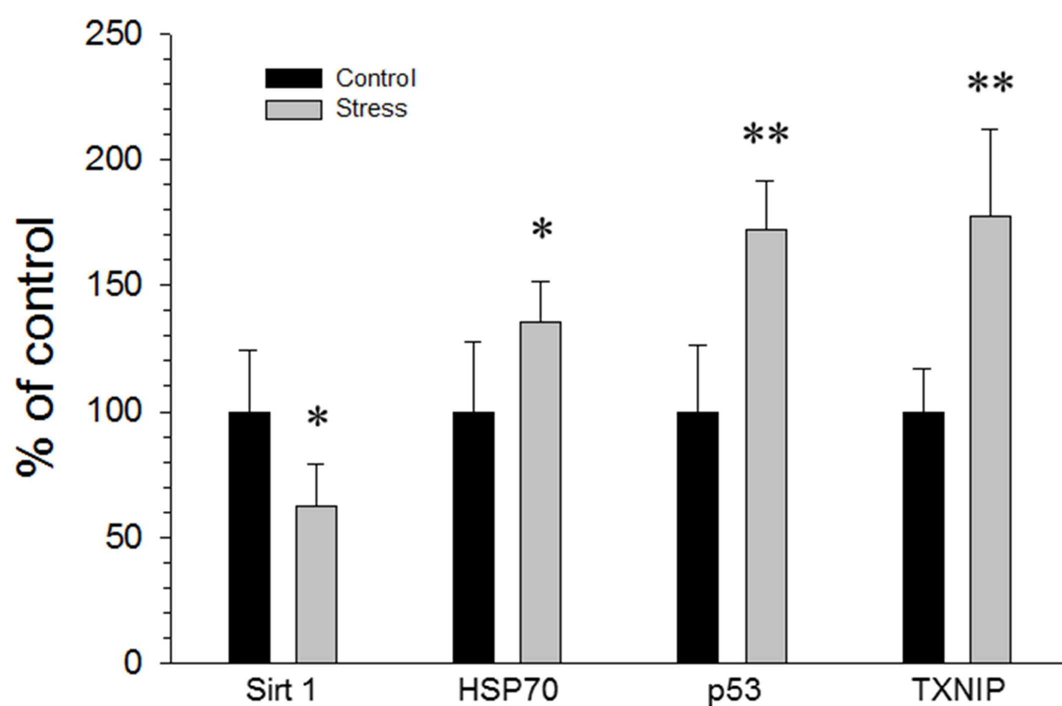

Body weight gain (g)
A
#
‡
‡
#
Control
S10d

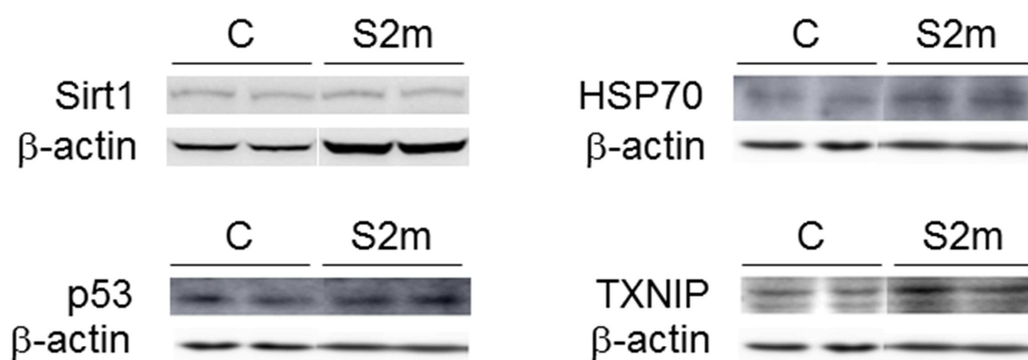

Adrenal weight (mg)
Vehicle
RU 486
B
‡
‡
Control
S10d

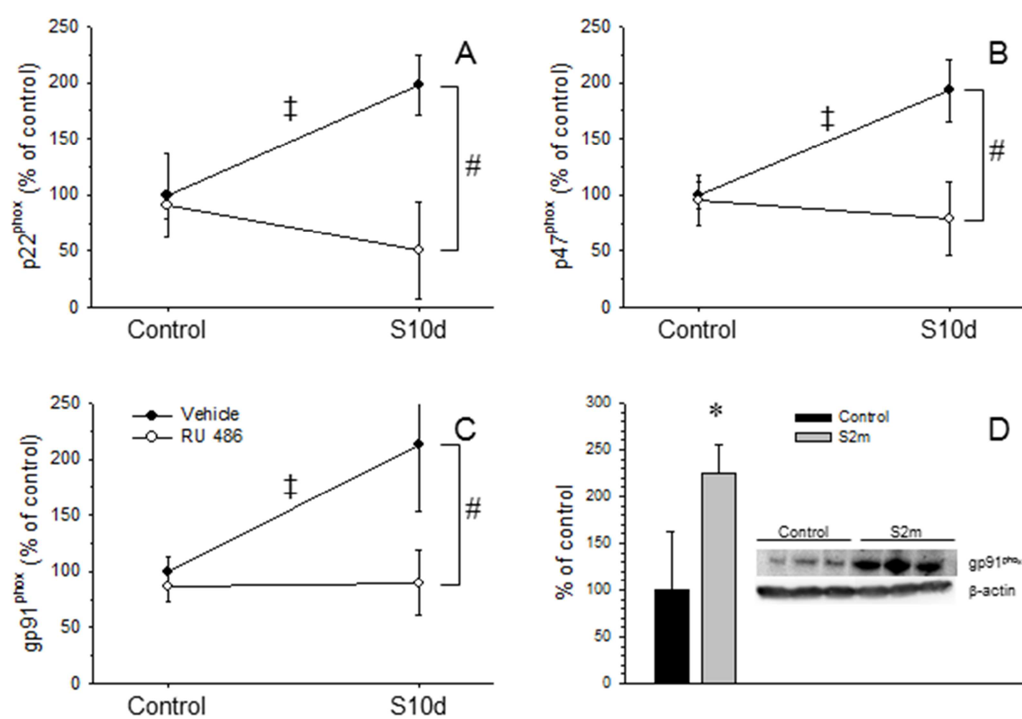

Corticosterone level (% of control)
C
Days

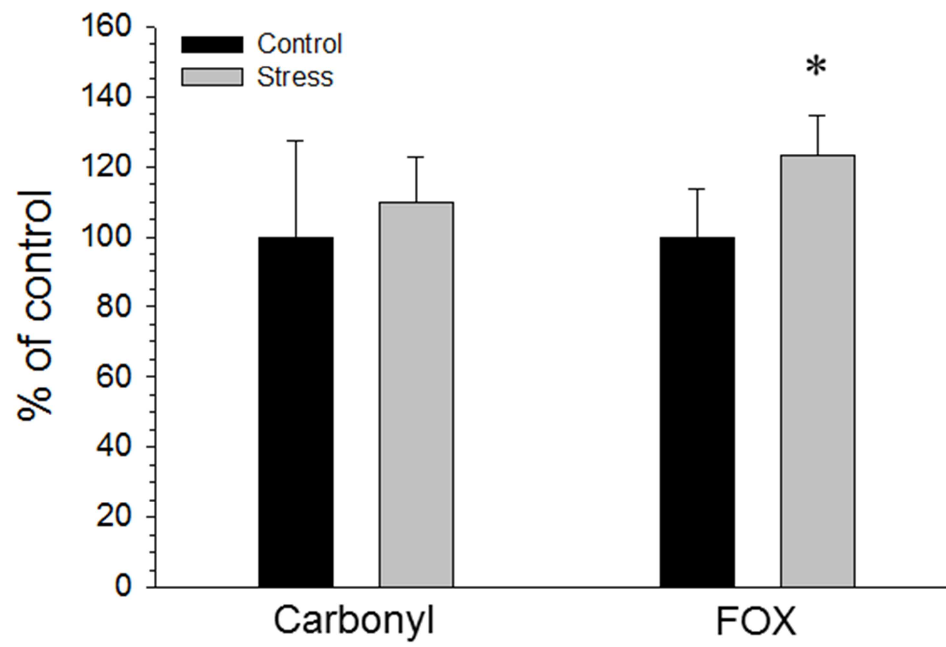


SIRT 1 (% of control)
HSP70 (% of control)
p53 (% of control)
TXNIP (% of control)
A
B
C
D
Control
S10d
‡
#
Vehicle
RU 486


250
200
150
100
50
0
% of control
Control
Stress
*
*
**
**
Sirt 1
HSP70
p53
TXNIP


C
S2m
Sirt1
β-actin
HSP70
β-actin
p53
β-actin
TXNIP
β-actin


A
B
C
D
p22phox (% of control)
p47phox (% of control)
gp91phox (% of control)
% of control
Control
S10d
Vehicle
RU 486
Control
S2m
gp91phox
β-actin
‡
#
*



160
140
120
100
80
60
40
20
0
% of control
Control
Stress
*
Carbonyl
FOX

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HIGHLIGHTS:

Chronic stress decreases the expression levels of sirtuin-1

Chronic stress increases the expression levels of p53 in the hippocampus.

Chronic stress increases ROS production through NADPH oxidase and TXNIP induction.

These effects are abolished by blocking the glucocorticoid receptor

Chronic stress could accelerate hippocampal aging.

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