Oxidative stress, neuroendocrine function and behavior in an animal model of extended longevity

Berry, A.

Chapter 3

Greater resistance to psychophysical stress in p66$^{Shc-/-}$ adult mice, a model of delayed aging

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Abstract

Evidence is mounting that reactive oxygen species (ROS) produced because of stressful challenges could interfere with the proper functioning of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in greater vulnerability to aging and neurodegeneration. Here we tested the hypothesis that p66$^{Shc-/-}$ mice, characterized by high resistance to oxidative stress and delayed aging, might be less susceptible to the effects of different stressful procedures involving an increase in ROS such as restraint stress or lipopolysaccharide (LPS) treatment. Although adrenocortical reactivity in response to restraint stress or LPS did not differ as a function of the genotype, a hyperdrive of the HPA axis was revealed following treatment with a synthetic glucocorticoid agonist. When measuring changes in hippocampal oxidative status following LPS, only wild-type (WT) subjects showed increased levels of F2-isoprostanes, an index of lipid peroxidation. At the same time, the neurotrophin brain-derived-neurotrophic factor was selectively increased in WT subjects, while levels of prostaglandin E_2 were reduced in the mutants. Overall, the greater resilience to stress-induced changes in the p66$^{Shc-/-}$ mutants might underlie the better health status and the greater longevity characterizing these mice.

Introduction

A number of studies have proposed that increased levels of reactive oxygen species (ROS) may represent a critical early event in normal aging and neurodegeneration (Finkel and Holbrook, 2000; Harman, 1956; 1998; Miquel, 1998).

The oxidative stress (OS) theory of aging has gained a strong experimental basis when Migliaccio and co-workers reported that deletion of the p66^{Shc} gene increased resistance to OS and led to a 30% increase in the lifespan of homozygous mutated mice (in a 129Sv/Ev background). Worth noticing, p66$^{Shc-/-}$ (KO) mice showed also a decreased incidence of some aging-associated pathologies (Francia et al., 2004; Migliaccio et al., 1999; Napoli et al., 2003). This protein acts as a specific redox enzyme in mitochondria, generating hydrogen peroxide (H_2O_2) in response to certain stimuli (Giorgio et al., 2007; Nemoto et al., 2006; Pinton et al., 2007). In addition, it has been recently shown that p66^{Shc}-generated ROS regulate the effects of insulin on energetic metabolism and that intracellular OS might accelerate aging, by favoring fat deposition and fat-related disorders, in mice (Berniakovich et al., 2008).

Evidence is mounting that stress and ROS might act synergistically to induce or exacerbate the neuronal decay associated with the neuropathology of several age-related neurodegenerative disorders. This interaction might involve an impairment in the negative feedback mechanism of the hypothalamic-pituitary-adrenal (HPA) axis, which is mediated by glucocorticoid receptors (GR) in the pituitary, hypothalamus and hippocampus (McEwen, 2000a). This hypothesis is supported by evidence indicating that inflammation and/or infection are stressful conditions characterized by increased generation of ROS. In this specific context, the HPA axis is constantly active, despite high levels of glucocorticoids (GC).

The causal factors underlying this peculiar change in HPA axis activity are still under study but might imply a role of ROS. Asaba and co-workers have shown that increased ROS levels prevent the low affinity GR-mediated repression of proopiomelanocortin (POMC) gene expression, thus impairing negative feedback, in vitro. From a mechanistic point of view, ROS might interact with GR in the pituitary corticotrophs, preventing their translocation from the cytoplasm to the nucleus (Asaba et al., 2004; Okamoto et al., 1999).

A well known glucocorticoid cascade hypothesis of aging (Landfield et al., 2007; Landfield et al., 1978; Sapolsky et al., 1985) states that the prolonged/excessive exposure to GC over lifetime, may result in a progressive loss of homeostasis leading to an allostatic load which is considered to play a pivotal role in the aging process. As for the nervous system, the hippocampus is considered to be particularly susceptible to circulating levels of GC and thus to the effects of the allostatic load. In fact this brain area contains high levels of GR (and mineralocorticoid receptors - MR), important for the HPA axis negative feedback, and for memory formation and storage. Effects of the overexposure to GC include disruption of synaptic plasticity, synaptic loss and atrophy of dendritic processes (McEwen, 2000b), which may be triggered by the decreased expression of neurotrophins and by inhibiting injury-induced sprouting (McEwen, 2000a; Smith et al., 1995).

P66$^{Shc-/-}$ mice are characterized by a deficient intracellular H_2O_2 production (Giorgio et al., 2007), thus it is conceivable that, following exposure to stressful stimuli, they might be less prone to ROS generation. This, in turn, could result in a more efficient GR-mediated negative feedback, preventing the deleterious consequences of a prolonged exposure to high levels of GC that can precipitate the aging process (Sapolsky, 1999). In line with the above hypothesis, we have previously shown that p66$^{Shc-/-}$ mice are characterized by smoother age-dependent changes in the behavioral profile, lower emotionality and better cognitive abilities at adulthood, in addition to a general healthier phenotype in aged individuals (Berry et al., 2007; Berry et al., 2008). Interestingly, KO subjects did also show increased basal levels of brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neuronal survival and differentiation, as well as in specific aspects of behavioral plasticity, emotionality and pain sensitivity (Berry et al., 2008; Cirulli et al., 2004; Thoenen, 1995). BDNF is negatively regulated by GC in the hippocampus (Barbany and Persson, 1992; Dwivedi et al., 2006). An intriguing hypothesis is that in the p66$^{Shc-/-}$ phenotype a reduced exposure to low levels of OS, starting during early life phases and throughout lifespan, might affect the set point of the HPA axis such that responses to stress are tightly and more efficiently tuned. In this context the increased BDNF basal levels, characterizing the p66$^{Shc-/-}$ mice, might be a direct consequence of the lower levels of circulating GC, in addition a more efficient HPA axis might result in an overall reduced allostatic load leading to an increased lifespan and healthspan.

High circulating GC can result in neurotoxicity through different mechanisms, including modifications in neuronal energy metabolism or via an increase in excitatory amino acids, such as glutamate, which may result in the induction of enzymes in-

volved in excitotoxic processes. Among these, cyclooxygenase 2 (COX-2) is the limiting factor in prostaglandin (PG) biosynthesis. COX-2 has been involved in excitotoxicity whereas under physiological conditions it plays a role in synaptic plasticity and long-term potentiation (LTP) (Chen et al., 2005; Sang et al., 2005). Interestingly, GC are known to down-regulate COX-2 expression (Yamagata et al., 1993), suggesting that the expression levels of this gene might be kept in balance by local concentration of glutamate and GC. Therefore, the p66$^{Shc-/-}$ mouse might be overall better equipped to face stressful challenges, and less exposed to the deleterious consequences of the aging process predicted by the "oxidative stress theory of aging" (Finkel and Holbrook, 2000; Harman, 1998).

The p66$^{Shc-/-}$ mutant mouse thus represents an appealing animal model to study the interaction between OS and the neuroendocrine system that might underlie the phenotypical traits of these long-living mutants.

The main aim of the present study was to assay the effects of stress-induced ROS levels in the homeostatic control of the neuroendocrine system of p66$^{Shc-/-}$ mutants and its relevance in the context of the aging process. Based on evidence suggesting a negative correlation between the oxidative cellular milieu and the functionality of GR, responsible for GC negative feedback, we hypothesized that p66$^{Shc-/-}$ mice, being less prone to ROS generation, would result in a more efficient activity of the HPA axis, especially under stressful challenging situations, eventually resulting in lower levels of circulating corticosterone (CORT).

We tested our hypothesis assessing the activation of the HPA axis as a result of challenges able to induce increased CORT levels and ROS generation such as acute restraint stress (psychophysical stress) or systemic inflammatory challenge (i.p. injection of lypopolysaccharide - LPS -) (Fontella et al., 2005; Madrigal et al., 2003; Miyake et al., 2005). The GC negative feedback of mutant subjects was specifically assessed under condition of OS by administering LPS and successively a potent GR agonist (dexamethasone - DEX -). We also performed a DEX-suppression-test (DST) (a DEX administration followed by a LPS injection) to test the ability of the HPA axis to escape from DEX suppression following an immunogenic challenge. The DST is a pharmacological test based on the ability of DEX, to inhibit the release of the endogenous GC, it is used in clinical practice to assay the sensitivity of the HPA axis and has been successfully extended to animal models involving HPA axis dysfunctions (Hatzinger et al., 1996).

In addition to circulating CORT levels, we measured hippocampal levels of BDNF, PGE_2, and isoprostane 15-F_{2t}-IsopP, a free radical-dependent lipid peroxidation product and OS marker. As a further index of oxidative status we measured the peripheral total reductive capacity (or anti-oxidant capacity, AOC), which has been recently reported to be reduced in patients affected by neurodegenerative diseases, including Alzheimer's and motor neuron diseases (Mandrioli et al., 2006; Minghetti et al., 2006).

Materials and Methods

Animals

Experimental subjects were adult (approximately 4-6 months of age) $p66^{Shc+/+}$ (WT) and $p66^{Shc-/-}$ (KO) male mice of the 129Sv/Ev strain, generated as previously described (Migliaccio et al., 1999) and bred in the animal facility of the Section of Behavioral Neuroscience at the Istituto Superiore di Sanità (Rome, Italy). Animals were kept under standard conditions: housed in an air-conditioned room (temperature 21 ± 1 °C, relative humidity 60 ± 10%) with a white-red light cycle (lights on from 08:30 to 20:30). Home cages were Plexiglas boxes (42 x 27 x 14 cm) with sawdust as bedding. Pellet food (Enriched Standard Diet purchased from Mucedola, Settimo Milanese, I-20019, Italy) and tap water were continuously available.

All the experiments were performed from 09:00 to 16:00 in a way such that CORT levels were at their trough in the morning and reached a peak in the evening. Different batches of animals were used for the restraint stress, LPS administration, GC negative feedback test (LPS-DEX) and DST challenges.

All experimental procedures were carried out in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

Restraint stress

Eight WT and 10 KO male mice underwent both an acute and a chronic restraint stress procedure. Experimental subjects were placed in a ventilated 50 ml conical Falcon tube (provided by hand made holes on the tube surface) for 30 minutes (Pacak and Palkovits, 2001) each day for 10 consecutive days at random times (Chen et al., 2005). On day 1 (acute stress challenge) and on day 10 (chronic stress challenge) animals were blood sampled by tail nick (approximately 200 µl) to assess CORT levels soon before the stress administration (baseline) and 30 min, 3 and 4 hrs after the onset of the stress procedure. All animals were pair housed and marked to allow a distinction among the subjects.

Lipopolysaccharide (LPS) treatment

On the basis of a dose-response pilot study previously run, both WT and KO subjects were injected i.p. with 1 mg/kg body weight (b.w.) of LPS (see Nguyen et al., 2004 for central effects of this LPS dose) or with vehicle alone (saline = SAL, injection volume 100 µl). The treatment groups were the following: WT-SAL (n=8); WT-LPS (n=8); KO-SAL (n=8); KO-LPS (n=8) and blood samples were collected by tail nick to assess CORT levels under basal conditions and two, 4 and 6 hrs following the treatment administration. In addition, rectal temperature was measured for each time point, right before the tail nick. Twenty-four hrs later rectal temperature was measured again, experimental subjects were sacrificed by decapitation, trunk blood was collected and both hippocampi dissected and immediately frozen. Successively blood

samples were used to assess both CORT levels and the anti-oxidant capacity (AOC), while hippocampal tissue was used to assess BDNF, 15-F_{2t}-IsoP and PGE_2 levels. Analysis of brain tissue was also performed on a group of WT and KO naïve mice.

LPS-Dexamethasone administration

To assess whether the negative feedback was differently affected in WT and KO mice by DEX administration, following the LPS-induced activation of the HPA axis, both WT and KO subjects were first i.p. injected with either LPS (1 mg/kg) or vehicle (100 µl) and 2 hrs later with either DEX (70µg/100g b.w. see Peinado et al., 2005) or vehicle (200 µl) (WT subjects: n = 4 for SAL-SAL, 6 for the SAL-DEX and 5 for both the LPS-SAL and for the LPS-DEX groups; KO subjects: n=4 for SAL-SAL, SAL-DEX and LPS-SAL groups and 5 for the LPS-DEX group). Blood samples were collected by tail nick at time 0 (baseline) and 4 hrs later (i.e. 4 hrs following the LPS and 2 hrs following the DEX injection) to assess peripheral CORT levels.

Dexamethasone-suppression-test (DST)

To test the ability of the HPA axis of WT and KO mice to escape from dexamethasone suppression following an immunogenic challenge experimental subjects were first injected either with DEX or vehicle (saline = SAL, 200 µl) and 2 hrs later with LPS (1mg/kg) or SAL (100 µl) (n= 5 for each group of treatment: DEX-SAL; DEX-LPS; SAL-LPS; SAL-SAL except for the KO-SAL-SAL group, n=4). Blood samples were collected by tail nick at time 0 (baseline) and 4 hrs later (i.e. 4 hrs following the DEX and 2 hrs following the LPS injection) to assess peripheral CORT levels.

Radioimmunoassay procedure

Blood samples (100 µl, approximate volume) were collected individually in potassium-EDTA coated tubes (1.6 mg EDTA/ml blood; Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at + 4°C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at - 20°C until further analysis. CORT was measured using a commercially available radioimmunoassay (RIA) kit containing ^{125}iodine labeled CORT; 5 µl of plasma were sufficient to carry out CORT measurement. Sensitivity of the assay was 0.125 mg/dl, inter- and intra-assay variation was less than 10 and 5%, respectively. (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

Measurement of anti-oxidant capacity (AOC)

Trunk blood was collected individually in anticoagulant-free vials 24 hrs following either SAL or LPS injection; at the same time, blood was also collected for the unhandled group. All samples were allowed to clot for 20 minutes at room temperature

and then centrifuged at 3000 rpm for 30 min at + 4°C. Serum was then stored at - 80°C until anti-oxidant capacity (AOC) determination.

The quantification of AOC in serum samples was determined using an assay, based on the reduction of Cu^{++} to Cu^{+} by the activity of all anti-oxidant species present in the sample as previously described (Minghetti et al., 2006). The reduced copper (Cu^{+}) forms a stable complex with bathocuproine that shows an absorption maximum at 490 nm. A standard curve of uric acid (1 to 1000 µM), as typical reducing agent, is used to calculate the anti-oxidant activity present in the sample. Data are expressed as µmoles/L of reducing power. The sensitivity was 22 µM of reductive capacity; intra- and inter-assay variability showed a coefficient of variance CV that was lower than 4%. Serial dilutions of each sample were analyzed in duplicate.

Brain dissection and BDNF, PGE_2 and15-F_{2t}-IsoP extraction

After sacrifice, both hippocampi were immediately dissected out, placed in plastic tubes, weighted, frozen on dry ice and stored at - 80 °C until metabolite extraction. A detailed procedure for prostaglandin E_2 (PGE_2) and F_{2t}-isoprostane (15-F_{2t}-IsopP) extraction has been described elsewhere (Minghetti et al., 2000). In brief, 200 µL of ice-cold Tris-HCl buffer pH 7.5 containing 10 µg/ml of the COX inhibitor indomethacin (stock solution 100x in ethanol) to avoid ex vivo PGE_2 synthesis, and 10 µM of the radical scavenger butylated hydroxy-toluene (stock solution 100x in ethanol) to avoid auto-oxidation, were added to each frozen sample, which was quickly thawed, homogenized with a Teflon pestle (Sigma) - 20 cycles in an ice bath - vigorously vortexed and centrifuged at 14000 rpm for 45 min at + 4 °C. Supernatants were collected and stored at - 80 °C until analysis. For BDNF extraction, tissue samples were rapidly thawed in the ice-cold buffer lysis, indicated in the manufacturer's instructions of the specific ELISA assay (Promega Corporation, Madison, WI), homogenized with a Teflon pestle - 20 cycles in an ice bath - vigorously vortexed and centrifuged at 14000 rpm for 45 min at + 4 °C. Supernatants were collected and stored at - 80 °C until analysis. Pellets were resuspended in 0.1N NaOH and total proteins quantification was assessed with the BCA protein assay kit (Pierce, Chemical Company, Ann Arbor, MI) with microplate procedure as detailed by the manufacturer.

PGE_2, 15-F_{2t}-IsoP and BDNF measurement

Measurement of PGE_2 and 15-F_{2t}-IsopP was performed in tissue extracts by high sensitivity colorimetric enzyme immunoassays (EIA kits, detection limit for PGE_2: 7.8 pg/mL, Assay Designs, Inc. Ann Arbor, MI; detection limit for 15-F_{2t}-IsopP: 2 pg/mL; Cayman Chemical, Ann Arbor, MI). According to the manufacturers, the cross-reactivity of the anti-PGE_2 antibody with 8-15-F_{2t}-IsopP was less than 0.25% and that of anti-15-F_{2t}-IsopP antibody for other prostaglandins was less than 1% (0.02% for PGE_2). The amount of BDNF in tissue extracts were measured (duplicate or triplicate) by a specific ELISA assay (Promega Corporation, Madison, WI). Detection limit of the assay was 7.8 pg/mL, and cross-reactivity of the anti-BDNF antibody with other related neurotrophic

factors (NGF, NT-3, and NT-4) was less than 3%. All measurements were run at least in duplicate for each sample. Results were expressed as pg/mg proteins.

Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with genotype and treatments as between-subjects factor and time blocks as within-subject, repeated measures, factor. Since data from DST did not follow a normal distribution, they were normalized by transforming them into the square root of the raw data. Post hoc comparisons were performed using the Tukey's test. In analyzing AOC data, this test was used in the absence of significant ANOVA effects according to the indications given by Wilcox (Wilcox, 1987).

Statistical analysis was performed using Statview II (Abacus Concepts, CA, USA). Data are expressed as mean ± SEM.

Results

Restraint stress

Fig. 1 shows that the restraint stress affected HPA axis activity in all mice (main effect of repeated measures: $F(3,48)=90.478$; $p<0.0001$) although it failed to reveal a difference between the two genotypes (main effect of genotype: $F(1,16)=0.940$; $p=0.3468$). In particular, a significant peak in CORT levels occurred in both conditions, 30 min after the onset of stress ($p<0.01$) (Pacak and Palkovits, 2001) (Fig.1 A and B), this increase being greater following chronic stress exposure ($p<0.01$, Fig. 1 B). In addition, a comparison between the acute and the chronic procedure revealed a more efficiently regulated negative feedback following chronic stress, regardless of genotype, as shown by lower values of CORT levels at 4 hrs compared to the acute condition ($p<0.05$).

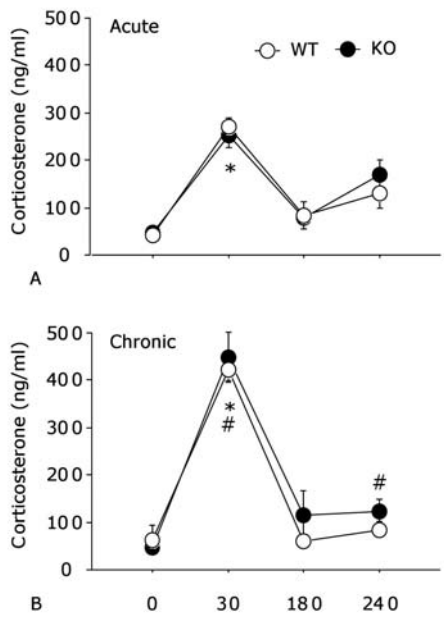

Figure 1 Restraint stress was equally effective to challenge the HPA axis both in WT and KO mice inducing a peak in CORT levels 30 minutes following the onset of stress both under acute (A) and chronic conditions (B). (B) The random chronic procedure prevented animals from habituation as shown by the greater 30-minutes-peak ($p<0.01$); chronic stress resulted overall in a more efficient negative feedback, regardless of genotype, as shown by lower CORT levels at 4 hrs ($p<0.05$). Data shown are mean ± SEM (n = 17 both WT and KO). * $p<0.05$; # $p<0.01$.

Lipopolysaccharide (LPS) treatment

CORT levels and rectal temperature were assayed in the same subject at different time points following LPS administration. No difference in CORT levels was found as a function of genotype ($F(1,28)=0.0300$; $p=0.9600$). LPS treated subjects were always characterized by significantly higher CORT levels than controls, except at 24 hrs (interaction between treatment and repeated measures: $F(4,112)=16.953$; $p<0.0001$, see Fig. 2 A). As for rectal temperature, no main effects of genotype and treatment were found ($F(1,28)=1.5280$; $p=0.2266$ and $F(1,28)=0.1890$; $p=0.6669$ respectively for genotype and treatment). An overall decrease in body temperature 6 hrs following LPS administration was found (mainly due to the KO group), and a successive return to basal values 24 hrs after the onset of stress (main effect of repeated measures: $F(4,112)=3.1890$; $p=0.0160$; post hoc comparisons $p<0.05$). Interestingly, KO mice were characterized by a higher basal body temperature (interaction between repeated measures and genotype: $F(4,112)=3.975$; $p=0.0447$; post hoc comparisons: $p<0.01$, see Fig. 2 B).

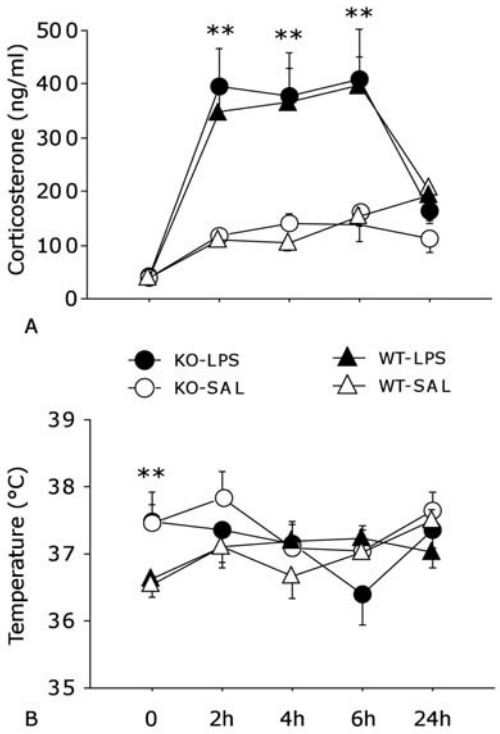

Figure 2 (A) CORT levels were increased in both WT and KO mice 2, 4 and 6 hrs following LPS administration. (B) Rectal temperature did not differ in the two genotypes as a result of LPS treatment. Interestingly KO subjects showed significantly higher basal values. Data shown are mean ± SEM (n = 8 for each group of treatment). Post hoc comparisons: ** for CORT levels: LPS vs. SAL, 2, 4 and 6 hrs following treatment administration; for rectal temperature: WT vs. KO basal values, $p<0.01$.

LPS-Dexamethasone administration

Both LPS and DEX affected CORT secretion regardless of genotype ($F(1,29)=34.685$; $p<0.0001$ and $F(1,29)=21.445$; $p<0.0001$, respectively). In particular, LPS increased, while DEX decreased CORT levels compared to vehicle. Nevertheless, no difference in CORT levels was found as a function of genotype ($F(1,29)=0.004$; $p=0.9494$, data not shown).

Dexamethasone-suppression-test (DST)

Both DEX and LPS affected CORT secretion regardless of genotype ($F(1,30)=19.641$; $p<0.001$ and $F(1,30)=105,302$; $p<0.0001$, respectively). In particular, DEX decreased, while LPS increased, CORT levels compared to vehicle (data not shown). When compared to their WT counterpart, KO showed a lower suppression of CORT levels as a function of DEX administration, regardless of LPS post-treatment (interaction among genotype, DEX, and repeated measures: $F(1,30)=4.738$; $p=0.0375$, see Fig. 3).

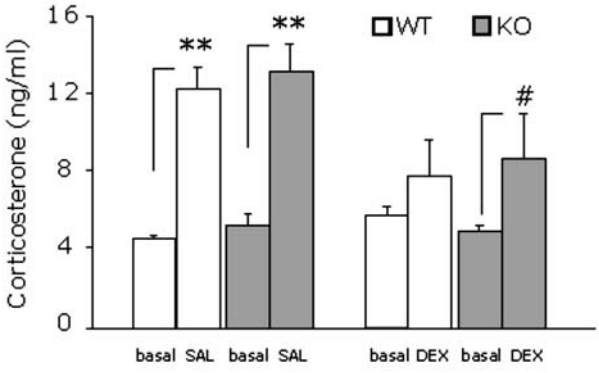

Figure 3 Escape from DEX suppression in KO mice: all subjects were first treated with DEX (or SAL) and successively challenged with LPS (or SAL). The figure represents exclusively the significant interaction among the first treatment (i.e. DEX or SAL), genotype and repeated measures. When compared to their WT counterpart, KOs showed a milder suppression of CORT levels as a function of dexamethasone administration ($p<0.0375$), regardless of LPS post-treatment. Experimental subjects represented in figure are pooled by either DEX or SAL treatment. Data shown are mean of the square root of raw data + SEM (n = 10 for each group of treatment except for the KO-SAL group, n = 8). Post hoc comparisons: ** effect of the manipulation i.e. (SAL) injection vs. basal values; # effect of DEX administration, KO-DEX vs. its control group (basal), $p<0.05$.

Measurement of anti-oxidant capacity (AOC)

Analysis of AOC indicated a clear tendency to an interaction between genotype and treatment, WT subjects reducing their AOC upon treatment with LPS ($F(2,24)=2.9400$; $p=0.0721$ see Fig. 4). Post hoc comparisons, performed on the interaction, revealed that, as a result of LPS challenge, WT mice show lower systemic anti-oxidant capacity (WT-LPS vs. WT-naïve and WT-SAL, $p<0.05$), which is likely to reflect a greater production of ROS.

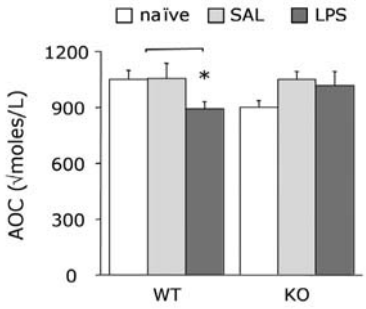

Figure 4 LPS administration reduced the AOC capacity of WT subjects (post hoc comparisons $p<0.05$). Data shown are mean + SEM. (WT-naïve = 6; WT-SAL = 5; WT-LPS = 4; KO-naïve = 5; KO-SAL = 5; KO-LPS = 5). Post hoc comparisons: * WT-LPS vs. WT-naïve and WT-SAL, $p<0.05$.

15-F_{2t}-IsoP measurement

The injection and handling procedures increased levels of 15-F_{2t}-IsoP only in WT subjects (interaction between genotype and treatment: $F(2,41)=3.877$; $p=0.0287$; post hoc comparisons: WT-SAL and WT-LPS vs. WT-naïve and WT-SAL vs. KO-SAL $p<0.01$, see Fig. 5 A).

PGE_2 and BDNF measurement

A significant interaction between genotype and treatment was found, PGE_2 being significantly decreased following SAL injection or LPS treatment, only in KO subjects ($F(2,38)=4.837$; $p=0.0134$; post hoc comparisons, $p<0.01$ see Fig. 5 B).

As for BDNF, a significant interaction between genotype and treatment was found ($F(2,37)=3.262$; $p=0.0496$). In particular, post hoc comparisons revealed that WT mice were characterized by increased levels of BDNF following treatment with LPS. As for basal levels, post hoc comparisons between KO-naïve and WT-naïve just missed statistical significance (Fig. 5 C).

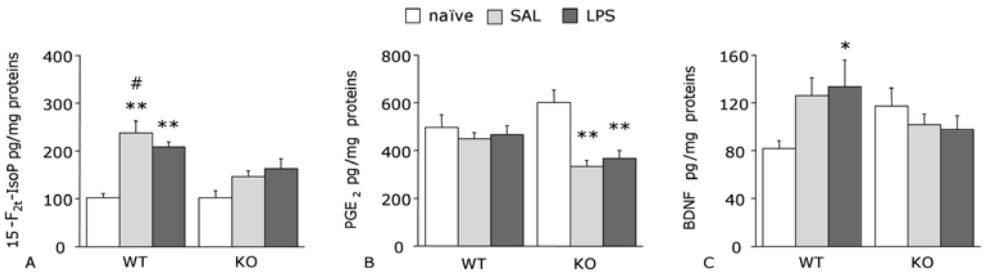

Figure 5 (A) Regardless of LPS administration, the injection and handling procedure increased the levels of 15-F_{2t}-IsoP only in WT subjects. (B) Mutant mice showed reduced levels of PGE_2 24 hrs following both of SAL or LPS administration while WT mice were not affected. (C) WT mice were characterized by increased levels of BDNF following treatment with LPS. As for basal levels, post hoc comparisons between KO-naïve and WT-naïve just missed statistical significance. Data are shown as mean + SEM. The number of samples used for PGE_2 and F_{2t}-IsoP was always equal to 8 except for PGE_2: WT-naïve n = 6, WT-SAL n = 7 and KO-SAL = 7, and for 15-F_{2t}-IsoP: KO-SAL n = 7. The number of samples used for BDNF was n = 6 for WT- and KO-naïve; n = 8 for WT- and KO-SAL and KO-LPS; n = 7 for WT-LPS. Post hoc comparisons: ** for PGE_2: KO-SAL and KO-LPS vs. KO-naïve; for 5-F_{2t}-IsoP: WT-SAL and WT-LPS vs. WT-naïve, and # WT-SAL vs. KO-SAL $p<0.01$; * for BDNF, WT-LPS vs. WT-naïve $p<0.05$.

Discussion

Findings from the present study indicate that p66$^{Shc-/-}$ mice do not show differences in their HPA axis reactivity to stressful challenges and in the GC negative feedback. However, they present a partial escape from DEX-induced suppression. Only WT subjects showed an increase of the oxidative status in the CNS following LPS or stress (LPS-related way of administration, i.p. injection) and this was accompanied by increased levels of BDNF. By contrast, PGE_2 - a powerful lipid mediator and a major product of COX-2, whose expression in cortical and hippocampal neurons is regulated by synaptic activity (Chen et al., 2002) and suppressed by GC (Yamagata et al., 1993) - was found to be reduced, only in the mutants. Overall, the greater resilience to stress-induced changes in the p66$^{Shc-/-}$ mutants might underlie the better health status and the greater longevity characterizing these mice (Berry et al., 2007; Berry et al., 2008; Migliaccio et al., 1999).

Acute and chronic restraint stress procedures were both able to activate the HPA axis. As for LPS administration, CORT levels were increased to a similar extent in all subjects, at least up to six hrs after LPS administration. The two genotypes also showed a similar pattern of rectal temperature in response to LPS. However, KO mice showed a higher basal core temperature, possibly suggesting a different set point for basal metabolism. This piece of data is in line with recent data on these mutants showing impaired thermo-insulation and increased metabolic rate (Berniakovich et al., 2008). More importantly, it enlarges the body of evidence supporting a relationship between changes in metabolism, energy homeostasis and longevity in higher organisms, which has been already proved for the lower ones (Bishop and Guarente, 2007; Kenyon, 2005).

Based on evidence showing that the functionality of GR may be negatively affected by ROS, we hypothesized that p66$^{Shc-/-}$ mice, being characterized by reduced levels of ROS following stress, could show a prompt and more efficient negative feedback, compared to WTs, as a result of stressful challenges. In the long run this would result in a reduced allostatic load, which could underlie the extended longevity and increased healthspan observed in the mutants. However, results form the LPS-DEX administration did not support our hypothesis since the negative feedback was similar in the two genotypes under conditions of oxidative stress (LPS). In addition, contrary to our expectations, p66$^{Shc-/-}$ mice were characterized by a moderate, although significant, escape of the HPA axis from suppression as revealed by the increase in CORT levels 4 hrs following DEX administration (DST).

The HPA axis is crucial in regulating the severity of age-associated diseases. Healthy ageing and longevity are likely to result from a lower propensity to mount inflammatory responses without compromising an acute response when, for instance, exposed to pathogens (Franceschi et al., 2007). Thus, in order to escape or to delay the major age-associated diseases and to extend longevity, it seems to be necessary to be able to cope successfully with the common diseases at young age (Franceschi et al., 2007). The rise in plasma GC levels, which usually occurs upon inflammation and/or infection, is necessary to prevent the overshooting of the immune function

(Gaillard, 2001; Wiegers and Reul, 1998) while hyporesponsiveness of the HPA axis has been associated to a higher susceptibility to autoimmune diseases (Bakker et al., 2000). Thus, the peculiar escape from suppression observed in the mutants at adulthood could indicate a better prepared system to cope with challenges the organism has to face throughout life.

In a previous study, we have shown that p66$^{Shc-/-}$ mice are characterized by an overall smoother age-dependent change in the behavioral profile (Berry et al., 2007). For instance, we observed both reduced pain sensitivity and emotionality, differences with wild-type subjects becoming more pronounced with age. Moreover mutant subjects were characterized by better physical performance, at senescence and, at adulthood, by more efficient cognitive strategies (as assessed in the Morris water maze test) associated to higher levels of BDNF and lower levels of OS markers in the hippocampus (Berry et al., 2007; Berry et al., 2008).

The mammalian brain is characterized by poor antioxidant defenses, high metabolic rate and reduced capacity for cellular regeneration resulting particularly susceptible to oxidative stress insults (Floyd and Hensley, 2002). In this study, we investigated the effects of LPS, which is able to challenge the HPA axis as well as to induce an inflammatory reaction that may affect CNS functionality, focusing our analysis on the hippocampus. This brain structure is particularly susceptible to GC-mediated degeneration given its unusually high concentrations of corticosteroid receptors (McEwen, 2000a; Sapolsky, 1999).

Our results indicate that central responses to stress in WT subjects include an increase in BDNF levels in the hippocampus, accompanied by increased oxidative status while KOs subjects appeared overall to be resilient. BDNF plays an essential role in the development, function and survival of neural populations, as well as in specific aspects of synaptic plasticity (Lewin and Barde, 1996; Thoenen, 1995; Yan et al., 1997). A number of in vivo and in vitro studies have reported different effects of LPS on BDNF synthesis and expression, although the existing body of evidence is still controversial (Elkabes et al., 1998; Miwa et al., 1997; Shaw et al., 2001). Here, the increase in BDNF protein observed in LPS-treated WT subjects may reflect neuroprotection following an insult. The lack of changes in BDNF levels, observed in KO subjects, could be interpreted as a greater basal neuronal protection against oxidative stress. In fact, in a previous study, mutant subjects have been shown to benefit from higher basal levels of BDNF which correlated with better cognitive abilities and lower emotionality (Berry et al., 2008). Worth noticing, WT subjects also showed a trend to increase BDNF levels as a consequence of the handling procedure (vehicle injection); we cannot exclude that this result might become significant using a larger number of experimental subjects. Stress and LPS are known to activate microglia and to modulate the secretion of proteins and/or the expression of various genes, including BDNF (Miwa et al., 1997), hence a specific increase in the levels of this neurotrophin may represent an essential step for WT subjects to buffer the potential deleterious effects of both glucocorticoids and LPS in the CNS.

A further observation of our study is the decreased levels of PGE_2 in the hip-

pocampus of p66$^{Shc-/-}$ mice following SAL/LPS challenge. We have already outlined the role of PGE_2, a molecule also involved in activity-dependent synaptic plasticity, like BDNF (Minghetti, 2007 and references therein). These two molecules may interact through COX, which has been shown to affect spatial learning and synaptic plasticity (Shaw et al., 2003). We have previously shown that adult mice carrying a targeted deletion of the p66^{Shc} gene showed better memory retention in the MWM and increased levels of BDNF in the hippocampus. In addition, the lack of this gene resulted in improved physical performance at senescence in the same spatial memory test (Berry et al., 2007; Berry et al., 2008). Thus, the lack of response in terms of BDNF synthesis and the decrease of PGE_2 levels in KO mice may well reflect a reduced neuronal activation of this genotype in response to a stressful challenge. We cannot exclude that the maintenance of slightly higher levels of circulating GC, as suggested by the DST test, could also lead to a more efficient suppression of PGE_2 in the mutant mice.

15-F_{2t}-IsoP (also named 8-epi-$PGF_{2\alpha}$) is a reliable in vivo index of free radical generation and lipid peroxidation, and it was previously found to be decreased upon high fatty acid diet in p66$^{Shc-/-}$ mice as compared to WT (Napoli et al., 2003). In vitro and in vivo studies have extensively reported that the p66$^{Shc-/-}$ mutation confers increased resistance to OS challenges in cultured cells as well as in peripheral tissues (Francia et al., 2004; Napoli et al., 2003; Trinei et al., 2002). Here we confirm and enlarge this body of evidence showing that mutant mice, regardless of treatment administration, were characterized by overall reduced central levels of oxidative stress (hippocampal levels of 15-F_{2t}-IsoP) and appeared to be not affected by the LPS administration or the handling procedure.

An alternative way to evaluate the degree of OS under specific conditions is the measurement of peripheral anti-oxidants, which comprise scavenging enzymes as well as many small anti-oxidant molecules, including vitamins glutathione and uric acid. Due to the complexity of peripheral anti-oxidant defenses and the induction of compensatory mechanisms under stressful conditions, the evaluation of variations in the levels of individual anti-oxidant species may not fully reflect the overall capacity of a subject to fight oxidant conditions. The measure of the total peripheral anti-oxidant defenses (AOC) has been recently proven as a useful index of the general health status and prognosis in patients affected by Alzheimer's disease, likely reflecting the extent to which vulnerable neuronal populations are protected from oxidant processes (Minghetti et al., 2006). In this study, we found that, differently from WT subjects, mutants did not diminish their anti-oxidant capacity following LPS treatment, which is consistent with the lower levels of hippocampal 15-F_{2t}-IsoP in these animals, and support the hypothesis of a reduced exposure of p66$^{Shc-/-}$ mice to OS. In addition, it is worth noticing that, although not significant, KO subjects showed a trend to increase their AOC, following both LPS and SAL, possibly related to an up-regulation of ROS scavengers following exposure to an oxidative stress stimulus (Pani et al., 2009).

It is important to note that in WT subjects, while hippocampal levels of 15-F_{2t}-Isop

were already affected by vehicle injection, peripheral changes of AOC only occurred upon LPS treatment. This can be explained taking into account that while vehicle injection is per se a stressful stimulus, which is elaborated centrally by the limbic system, LPS administration initially causes a peripheral response which is likely to affect peripheral oxidative status.

Taken together, these data suggest that p66$^{Shc-/-}$ mice might be characterized by a more efficient homeostatic control and by better abilities to cope with changes in the internal milieu. This may be achieved by two possible pathways acting synergistically: one involving an OS-resistance mechanism, the other, a fine regulation of the HPA axis, a process independent from the GC negative feedback, as initially hypothesized, that in turn, may be related to a less harsh oxidative milieu.

The aging process is related to a low-grade, chronic state of inflammation defined as inflammaging (Franceschi et al., 2007). A reduced GC anti-inflammatory activity may contribute to such state (Landfield et al., 2007). Thus, a moderate increase in GC levels, as shown by p66$^{Shc-/-}$ mice following the DST test, may be related to the previously observed delay in aging and the better health status characterizing this animal model (Nelson et al., 1995; Sabatino et al., 1991; Sapolsky, 1995).

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