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Sustained Hippocampal Neurogenesis in Females Is Amplified in P66$^{Shc-/-}$ Mice: An Animal Model of Healthy Aging

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ABSTRACT: Aging is accompanied by poor learning and memory abilities and by decreased hippocampal neurogenesis, a process that is also modulated by oxidative stress (OS). P66^{Shc} has recently emerged as a novel mammalian gerontogene able to affect healthspan during aging. Deletion of this gene in mice leads to reduced OS accompanied by decreased incidence of age-related pathologies and reduced signs of behavioral aging. We hypothesized that p66$^{Shc-/-}$ mutants might show increased neurogenesis in the hippocampus, a brain region involved in learning and memory processes. To this aim, granule cell number, proliferation, neuronal differentiation, and cell death were assessed in the hippocampus in senescent p66$^{Shc-/-}$ [knock out (KO)] and p66$^{Shc+/+}$ [wild type (WT)] male and female mice. Spatial learning abilities and spontaneous activity were also investigated in a multifunctional behavioral system—IntelliCages. The behavioral analysis revealed that females in general perform better in spatial learning tasks, with genotype effects being apparent in the activity pattern only. Likewise, all females showed increased neuronal differentiation, whereas increased proliferation was found only in those belonging to the p66$^{Shc-/-}$ genotype, indicating that they might be protected from precursor cell loss. The number of dying cells was not affected by genotype or sex; however, all KO mice showed less granule cells than WT. Overall, our data suggest that hippocampal function is protected in the female gender at older age, an effect amplified by reduced OS in the p66$^{Shc-/-}$ mutant. © 2012 Wiley Periodicals, Inc.

KEY WORDS: transgene; stereology; oxidative stress; gender; learning

INTRODUCTION

Life expectancy in the 20th century has increased greatly in Western Societies, but it has been paralleled by a progressive rise in the incidence of age-related diseases, including neurodegeneration and dementia, which interfere with the quality of life. Aging is a multifactorial degenerative process that strongly impacts the endocrinology and biochemistry of the brain (Bishop et al., 2010). The hippocampus is a brain region playing a pivotal role in cognitive processes, and aging leads to important changes in neurotransmission as well as in the expression of trophic factors and their receptors [e.g., brain-derived neurotrophic factor (BDNF) and tyrosine kinase B (TrkB); Lee and Son, 2009). In addition, the aging hippocampus is affected by oxidative stress (OS), possibly influencing neuronal loss and atrophy, increased production of inflammatory mediators, and increased vasculopathy (reviewed in Miller and O'Callaghan, 2005 and in Smith et al., 2005).

The physiological decay characterizing aging occurs at a life stage when proliferation and integration of adult-born neurons in the hippocampus have become a rare event. Even few young neurons, however, are thought to contribute to the maintenance of hippocampal homeostasis, representing a mechanism that can modify neuronal networks in response to external stimuli (Lee and Son, 2009; Snyder and Cameron, 2012). Within the hippocampus, the process of generating new granule cells (neurogenesis) occurs in local microenvironments—neurogenic niches—localized around blood vessels in the subgranular zone (Palmer et al., 2000; Alvarez-Buylla and Lim, 2004). The inhibition of neurogenesis has been related to impairments in spatial and contextual memory, suggesting a role for adult neurogenesis in maintaining regular memory function (Villeda et al., 2011). Neurogenesis reaches a peak at puberty and declines dramatically thereafter. This decline, which has been observed in most mammals, may relate to age-related decreases in performance in learning and memory tests (Gould et al., 1999; Amrein and Lipp, 2009; Ben Abdallah et al., 2010).

A number of studies have proposed that OS and the increased levels of reactive oxygen species (ROS) may represent a critical early event in normal aging and neurodegeneration associated with cognitive impairment (Floyd, 1999; Finkel and Holbrook, 2000). In addition, oxidative insults and inflammation might affect the plastic potential of the hippocampus related to neurogenesis (Bastos et al., 2008; Russo et al., 2011).

The mammalian brain is characterized by a high susceptibility to OS due to poor antioxidant defenses

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(Floyd, 1999; Finkel and Holbrook, 2000). $P66^{Shc}$ has recently emerged as a novel gerontogene encoding for a redox enzyme that has been hypothesized to accelerate aging in mice by favoring the generation of ROS (H_2O_2) on metabolic (insulin) signaling (Migliaccio et al., 1999; Berniakovich et al., 2008; Giorgio et al., 2012). Targeted deletion of this gene in the 129Sv/Ev mice results not only in an extension of lifespan of about 30% (Migliaccio et al., 1999) but also, and more importantly, in better cognitive abilities at adulthood in a spatial memory task and improved physical performance at senescence (Berry et al., 2007, 2008, 2010).

In this context, we sought to investigate whether the lack of $p66^{Shc}$ might also affect neurogenesis as a possible mechanism underlying the healthspan of mutant mice. To test this hypothesis, knock-out (KO; $p66^{Shc-/-}$) and wild-type (WT; $p66^{Shc+/+}$) male and female mice of aged 21–24 months were examined for cell proliferation, neuronal differentiation, and cell death. Measurements of total granule cell number and mossy fibers volume were obtained to evaluate sustained alterations within the hippocampal formation. In addition, we investigated brain functions in both male and female 24-month-old mice by testing them for cognitive abilities in a spatial memory task. We have previously shown that both WT and KO senescent mice are poor learners in the Morris water maze (MWM; Berry et al., 2008). Consequently, we here used a test apparatus characterized by reduced physical load allowing testing the animals in a social environment, the IntelliCages.

A large body of evidence suggests that gender differences exist in the rate of aging. In particular, estrogens appear to attenuate age-related cognitive decline in female subjects of species from rodents to primates (Gibbs and Gabor, 2003). This effect has been suggested to be related to neuronal protection mediated by an anti-inflammatory action (Bruce-Keller et al., 2000; De Nicola et al., 2009). As very few studies have investigated gender differences in hippocampal neurogenesis, we compared the effects of lack of the $p66^{Shc}$ gene in both senescent male and female mice.

MATERIALS AND METHODS

Animals

$P66^{Shc-/-}$ mice (Migliaccio et al., 1999) and wild-type mice with background strain 129Sv/Ev were bred and maintained in the animal facility of the Section of Behavioral Neuroscience, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy. Animals were housed in groups of three per cage in 42 × 27 × 14 cm^3 Plexiglas cages with metal tops and sawdust as bedding in an air-conditioned room (temperature 21 ± 1°C and relative humidity: 60% ± 10%) with lights on at 7 a.m. until 7 p.m. 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). As for behavioral assessment, 29 animals were tested in the IntelliCages (females: 7 WT and 8 KO; males: 7 WT and 7 KO), whereas a different batch of 26 mice (females: 9 WT and 4 KO; males: 8 WT and 5 KO; Table 1) was used for histological analysis. All experimental subjects were aged 21–24 months.

TABLE 1.

Morphological Measurements in the Dentate Gyrus

	N	Total granule cell	SD granule cells	CE granule cells	Mossy fiber field (mm^3)
Genotype					
$P66^{Shc-/-}$	9	475,802	61,133	0.08	331.0
$P66^{Shc+/+}$	17	531,558	45,927	0.07	345.0
Gender					
F $p66^{Shc-/-}$	4	457,571	92,065	0.07	336.0
M $p66^{Shc-/-}$	5	490,387	22,789	0.08	327.0
F $p66^{Shc+/+}$	9	532,641	48,114	0.06	343.0
M $p66^{Shc+/+}$	8	530,341	46,608	0.07	348.0

Estimates of total granule cell number using the optical fractionator method; volume of mossy fiber terminal field measured with Cavalieri. Abbreviations: SD, standard deviation; CE, coefficient of error; F, females; M, males.

Histology

Mice were deeply anesthetized using Pentobarbital (50 mg/kg) and perfused transcardiacally with phosphate buffered saline (pH 7.4), followed by 0.6% sodium sulfide solution (5.85 g of Na_2S and 5.95 g of $NaH_2PO_4 \times 2H_2O$ in 1,000 ml distilled water) and cold 4% paraformaldehyde containing 15% saturated picric acid. Brains were removed, divided into the hemispheres, and postfixed in the fixative at 4°C overnight. Right hemispheres were cryoprotected with 30% sucrose, frozen and cut sagitally into 40-μm sections. Series of every 10th section were collected and stored in a cryoprotectant solution at −20°C until further processing. Left hemispheres were dehydrated and embedded in 2-hydroxyethylmethacrylate (HEM; Technovit 7100, Kulzer GmbH, Bern, Switzerland) according to manufacturer's instruction and as described before (Amrein et al., 2004). Embedded tissue was cut horizontally into 20-μm sections and collected in series of every sixth section.

Nuclear and Mossy Fiber Stain

One series each of the 20-μm HEM-embedded left hemisphere sections and of the 40-μm cryostat sections of the right hemispheres were mounted and Giemsa stained as described before (Klaus et al., 2009). In brief, sections were incubated for 10 min in Giemsa stock solution (diluted 1:10 in 67 mmol of KH_2PO_4; Merck, Darmstadt, Germany) at 60°C, differentiated in 1% acetic acid, dehydrated, and cover slipped using Eukitt. A second series of HEM-embedded tissue was stained with Timm's silver sulfide method to visualize mossy fibers (Schwegler and Lipp, 1983). For this, mounted sections were devel-

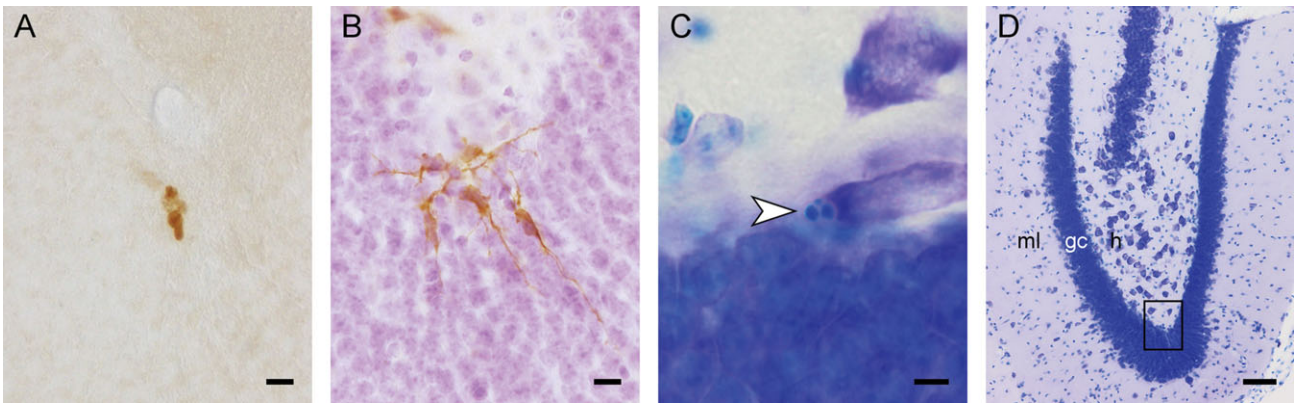

FIGURE 1. Immunohistochemical and nuclear stainings in adjacent sections of a female KO mouse. We found proliferating cells (Ki67-positive; A) and young cells of the neuronal lineage (DCX-positive; B) in the crest region of the Giemsa-stained dentate gyrus (box in D). Rare dying cells (arrow in C) can be identified in the subgranular layer by their intensively stained nuclear fragments. Scale: A: 50 µm; B and C: 10 µm; and D: 5 µm. Abbreviations: ml, molecular layer; gc, granule cell layer; h, hilus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oped at 37°C in darkness for ~60 min in a 6:3:1 mixture of gum solution [33.33 g of Gummi arabicum (Fluka, Buchs, Switzerland) and 0.03 g of thymol (Merck, Darmstadt, Germany) in 100 ml of distilled water], hydroquinone solution [5 g of hydroquinone (Sigma-Aldrich, Steinheim, Germany) in 100 ml of distilled water], and citrate buffer (2.55 g of citric acid and 2.53 g of trisodium citrate dihydrate in 10 ml of distilled water) containing silver nitrate [0.5 ml of 17% $AgNO_3$ (Fluka) for 100 ml developer solution]. Afterward, slides were rinsed in tap water followed by two rinses in distilled water and subsequently fixed in 1% sodium thiosulfate for 1 min. Finally, the slides were dehydrated and then cover slipped using Eukitt.

Immunohistochemistry: Doublecortin and Ki67 Staining

The immunohistochemical stainings were performed free floating on every fifth section, using two equidistant series of sections, for doublecortin (DCX) and Ki67. Sections were washed before every step with Tris-Triton (0.05% Triton in tris-buffered saline (TBS), pH 7.4) until incubation with the primary antibody, afterward with TBS (pH 7.4) only. For the DCX epitope retrieval, sections were treated in the microwave (~600 W) in 1:10 citrate buffer (Target Retrieval Solution, DAKO, Denmark) in distilled water for 3 min and incubated in 0.6% H_2O_2 in Tris-Triton for 15 min to block endogenous peroxidase activity. For Ki67 epitope retrieval, sections were incubated in the same citrate buffer and treated in a water bath for 40 min at 94°C. Thereafter, sections were preincubated for 60 min at room temperature in TBS containing 2% serum [normal rabbit serum for DCX and normal goat serum for Ki67], 0.25% Triton, and 0.1% bovine serum albumin, followed by incubation overnight at 4°C with the primary antibodies in preincubation solution [polyclonal goat IgG, 1:1,000 (Santa Cruz Biotechnology) or polyclonal rabbit NCL-Ki67p, 1:5,000 (Novocastra)].

Sections were rinsed and incubated with the secondary antibody (1:300 anti-goat for DCX and 1:1,000 anti-rabbit for Ki67; Elite ABC Kit, Vectastain) at room temperature. Then, sections were incubated in avidin–biotin complex (Elite ABC Kit, Vectastain) according to the manufacturer's instruction and stained with diaminobenzidine.

Sections were mounted in the correct anatomical order on slides. DCX-stained sections were counterstained with a hematoxylin solution (Fluka) for 4 min, dehydrated, and then cover slipped with Eukitt.

Measurements

Prior to the counting procedure, all series of sections were coded and counts were performed blindfolded.

Proliferating Cells and New Differentiating Neurons

Because of the low numbers of positive cells, Ki67 (Fig. 1A) and DCX (Fig. 1B), immunopositive cells were counted exhaustively in the dentate gyrus of every fifth section using a 63× oil-immersion lens (N.A. 1.25). To avoid overestimation, positive cells in the bottom focal plane were not considered. Cells were counted separately in the suprapyramidal (SP) and infrapyramidal (IP) blades and in the crest region. Total number of positive cells was estimated by multiplying cell counts by 5.

Pyknotic Cells

Pyknotic cells (Fig. 1C) were counted exhaustively in every sixth HEM-embedded, Giemsa-stained section and in every 10th Giemsa-stained cryostat section using a 100× oil-immersion objective (N.A. 1.30). Pyknotic cells were characterized by strong and homogenously stained nuclei or nuclear fragments in the lower one-third of the dentate granule cell layer and up to two cell layers into the hilus (Amrein et al., 2004). Again,

cells in the bottom focal plane were excluded from the count. An estimate of total pyknotic cell numbers was obtained by multiplying cell counts by section sampling fraction, that is, by 6 and 10, respectively.

Estimation of Total Granule Cell Number

Total granule cell number was estimated using the optical fractionator method (West et al., 1991) of StereoInvestigator software (MicroBrightField, Williston, ND). Cells (Fig. 1D) were counted through a 100× oil-immersion lens (N.A. 1.30) in every sixth HEM-embedded, Giemsa-stained section within a counting frame of 10 × 10 μm^2 and dissector height of 10 μm. The *x*- and *y*-step sizes between sampling locations were 120 μm. Estimation of the total number of granule cells (*N*) of one hemisphere is calculated by the following formula:

$$N = \sum Q^- \times (t/h) \times (1/\text{asf}) \times (1/\text{ssf}),$$

where Q^- = total number of counted cells, t = section thickness, h = dissector height, asf = area sampling fraction = $a(\text{frame})/a(x - y \text{ step})$, and ssf = section sampling fraction. Section thickness was measured at every sixth sampling location, and number-weighted section thickness (Dorph-Petersen et al., 2001) was used for *t*.

Volume Measurement of Mossy Fiber Terminal Field

The volume of the mossy fibers in the dentate gyrus was measured using the Cavalieri method (StereoInvestigator software; MicroBrightField) with a 10× lens in every sixth HEM-embedded, Timm-stained section. A grid size of 45 × 45 μm^2 was used for intra-IP and 90 × 90 μm^2 for SP and hilus terminal fields.

Spatial Learning and Spontaneous Behavior in the IntelliCages

The IntelliCage (NewBehavior AG, Zürich, Switzerland) is an apparatus for automatic monitoring of mouse behavior in the home cage. This system is able to score the activity and learning behaviors of every individual living in a social group as every animal is identified by a subcutaneous transponder. An IntelliCage consists of a large acrylic rat cage (20.5 cm high, 58 × 40 cm^2 at the top, and 55 × 37.5 cm^2 at the base, Model Tecniplast 2000) with four walls separating each corner from the center so that they form four identical chambers, with two doors each, to access to water. Doors are programmable and might be left opened for free exploration or opened following a visit to the corner (see Krackow et al., 2010). Twenty-nine animals were housed such that 15 females were grouped in one and 14 males in another group for 14 days. Five days before being moved to the IntelliCages, each animal was injected with a subcutaneous transponder (T-IS 8010 FDX-B; Datamars SA, Switzerland). During the entire experiment, food was freely available. During the first 7 days, the animals were habituated to the IntelliCage environment, which became their home cage, and were allowed to freely explore all the corners, and the individual preference for a corner was established. Once the preference was established for each subject, animals were allowed to drink only from the corner opposite to the preferred one (Days 8–11, spatial learning phase). Successively, all subjects underwent a 3-day spatial learning reversal phase where the only corner available for drinking was the one preferred during the adaptation phase (Days 12–14).

Behavioral parameters scored were as follows: frequency of corners' visits during the free exploration phase as an indication of the general exploratory activity, and percent errors during the place-learning and reversal phase tasks as measures of cognitive performance (see also Krackow et al., 2010, and references therein).

Statistical Analysis

Behavioral data were analyzed using analysis of variance (ANOVA), considering genotype and sex as between-subject factors and days as a repeated measures/within-subject factor. Post hoc comparisons have been performed using Tukey's test. Cell counts, weights, and volumes were analyzed with a two-way ANOVA, with genotype and sex as between-subjects factors and age (21 and 24 months) as covariate. The same analysis was used with normalized cell counts, that is, numbers of Ki67-positive cells, DCX-positive cells, and pyknotic cells expressed as a percentage of the estimated total granule cell number. Correlations of cell counts (Ki67, DCX, and pyknotic cells) and total granule cell numbers were tested using two-tailed Pearson's *r*.

The quality of quantitative estimates obtained from design-based stereological methods was calculated using the coefficient of error (CE) and a conservative $m = 0$ approach (Gundersen et al., 1999; Slomianka and West, 2005). Coefficient of variance (CV) was calculated as standard deviation of the population divided by the population mean. CE^2/CV^2 values < 0.5 indicate that only little of the variance observed in the sample is generated by the estimation procedures.

Cell count data (the number of apoptotic cells in the granule cell layer (GC), along with the number of Ki67-positive and DCX-positive cells in the GC, SP blade, IP blade, and crest region) were used in a principal component analysis (PCA). This is a data-reduction method in which a large number of variables can be re-expressed as a new set of variables that are uncorrelated. The new variables are termed the principal components (PCs), and the key feature is that the first few PCs usually account for the majority of the variation in the raw data, and ideally, these first few components are related to the experimental factors. PCA is also useful for visualizing multivariate relationships and as a quality control method to check for multidimensional outliers or usual clustering in the data, which might be due to technical factors such as differences between batches, litters, and day of data collection.

Statistics were performed with SPSS 17.0 (SPSS, Chicago, IL) for cell counts and with Statview II (Abacus Concepts, CA) for the behavioral analysis. PCA was conducted in R (www.r-project.org) using the made4 package.

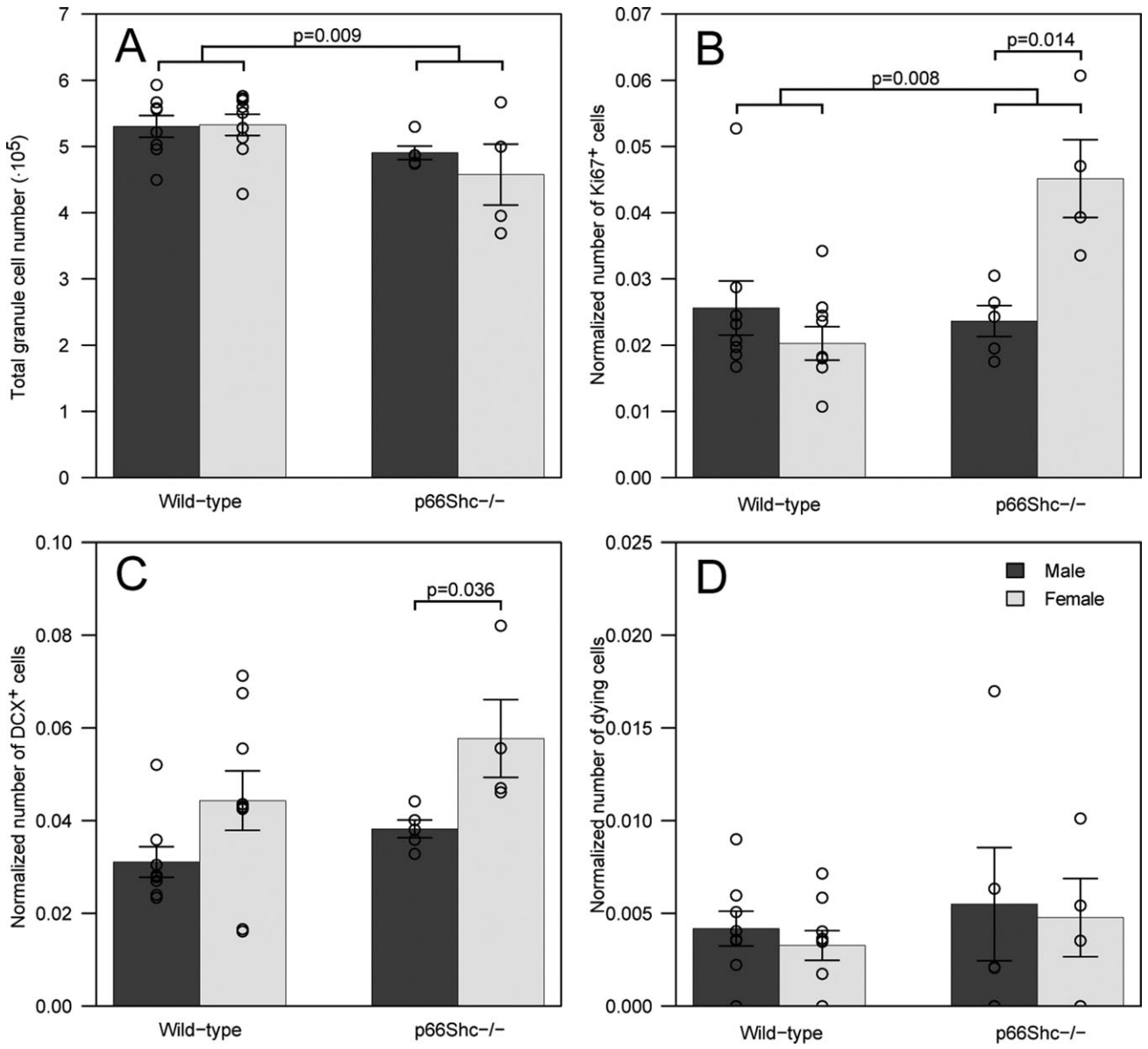

FIGURE 2. The total number of granule cells is reduced in KO mice (A). Proliferation is increased in female KO mice when compared with males; overall, KO mice show increased proliferation than WT (B). Neuronal differentiation is increased in female KO; overall, females have more young neurons than males ($P = 0.015$; C). There is no difference in cell death between genotypes or gender (D). Data in B–D are given as a percentage to total granule cell number. Error bars indicate ± SEM; individual data points are indicated.

RESULTS

Morphological and Cellular Alterations Due To Genotype, Sex, and Age

Senescent $p66^{Shc-/-}$ mice were leaner [lower body weight: $F(1,24) = 6.27$; $P = 0.021$] and with smaller brains [$F(1,24) = 7.8$; $P = 0.011$] than WT mice. Overall, females had smaller brains [$F(1,24) = 7.83$; $P = 0.011$] and were leaner [$F(1,24) = 16.3$; $P = 0.001$] than males, but showed an increased relative brain weight (brain weight/body weight) than males [$F(1,24) = 14.87$; $P = 0.001$]. We did not find a genotype effect in the relative brain weight and no genotype by gender interaction for brain or body weight. Within KO mice, females had significantly lower body weight [$F(1,7) = 21.94$; $P = 0.003$] but not brain weight [$F(1,7) = 2.5$; $P = 0.164$] than males. Regarding aging processes, we observed a decrease in body and brain weight in all animals aged between 21 and 24 months. However, all neurogenesis-related cell estimates did not differ between these ages (data not shown). The total number of granule cells was significantly lower in KO than in the WT mice [$F(1,24) = 8.25$; $P = 0.009$; Fig. 2A]. Despite this difference, the volume of mossy fibers terminal fields, that is,

TABLE 2.

Neurogenesis-Related Cell Numbers

	N	Ki67 (SD)	$\frac{CE^2}{CV^2}$ Ki67	DCX (SD)	$\frac{CE^2}{CV^2}$ DCX	Dying cell (SD)	$\frac{CE^2}{CV^2}$ Dying
Genotype							
$P66^{Shc-/-}$	9	154 (56)	0.13	225 (93)	0.12	22 (11)	0.46
$P66^{Shc+/+}$	17	119 (47)	0.15	199 (78)	0.11	21 (13)	0.30
Gender							
F $P66^{Shc-/-}$	4	202 (44)	0.41	272 (131)	0.11	19 (13)	0.24
M $P66^{Shc-/-}$	5	116 (27)	0.31	187 (18)	1.79	24 (9)	0.71
F $P66^{Shc+/+}$	9	107 (37)	0.20	229 (85)	0.12	24 (16)	0.25
M $P66^{Shc+/+}$	8	134 (55)	0.12	166 (56)	0.15	16 (9)	0.48

Estimated total cell numbers of proliferating (Ki67) cells, young neurons (DCX), and dying cells of one hemisphere. Abbreviations: SD, standard deviation; CE, coefficient of error; CV, coefficient of variance; F, females; M, males.

the main output structures of granule cells, did not differ between genotypes [$F(1,24) = 1.6$; $P = 0.221$; Table 1].

Specific Effects of Genotype and Gender on Cell Proliferation and Neuronal Differentiation, but No Effect on Cell Death

When cell proliferation was analyzed, KO mice were found to harbor more proliferating cells than WTs. This effect was marginal for total number of proliferating cells. However, when values were normalized to total granule cell number, the effect was significant [main effect of genotype: $F(1,24) = 4.35$; P_{total} 0.05 and $F(1,24) = 8.55$; $P_{normalized} = 0.008$; see Fig. 2B]. This effect is mainly due to $p66^{Shc-/-}$ females having more proliferating cells when compared with males of the same genotype [$F(1,7) = 16.65$; $P_{total} = 0.006$ and $F(1,7) = 11.67$; $P_{normalized} = 0.014$], apparent also by a genotype by gender interaction of $F(1,24) = 9.62$; $P_{total} = 0.005$ and $F(1,24) = 11.63$; $P_{normalized} = 0.003$ (Table 2).

As for neuronal differentiation, the number of young cells of the neuronal lineage did not differ between WT and KO mice [genotype main effect: $F(1,24) = 0.89$; $P_{total} = 0.357$ and $F(1,24) = 2.94$; $P_{normalized} = 0.101$]; however, a significant interaction between genotype and sex indicated that $p66^{Shc-/-}$ females are characterized by more young neurons than $p66^{Shc-/-}$ males [$F(1,7) = 3.09$; $P_{total} = 0.129$ and $F(1,7) = 7.29$; $P_{normalized} = 0.036$; Fig. 2C]; WT male and female subjects did not differ between each other [$F(1,15) = 2.57$; $P_{total} = 0.131$ and $F(1,15) = 2.48$; $P_{normalized} = 0.138$; Table 2]. Pooled females ($p66^{Shc-/-}$ and $p66^{Shc+/+}$) show significantly more young neurons in the dentate gyrus than males [main effect of sex: $F(1,24) = 5.18$; $P_{total} = 0.033$ and $F(1,24) = 7.04$; $P_{normalized} = 0.015$].

Evaluation of the number of dying cells did not differ between the HEM-embedded and cryostat sections. The mean CE was lower in the HEM-embedded material, and thus, data derived from the HEM-embedded material were used for the analysis. The number of dying cells showed a great variability in both genotypes, was independent from sex, and overall did not differ between genotypes [$F(1,24) = 0.006$; $P_{total} = 0.939$ and $F(1,24) = 0.36$; $P_{normalized} = 0.557$] and gender [$F(1,24) = 3.03$; $P_{total} = 0.61$ and $F(1,24) = 0.27$; $P_{normalized} = 0.608$; Fig. 2D and Table 2].

Overall, the number of proliferating cells, dying cells, and young neurons did not correlate with each other. Total number of granule cells did not correlate with any of the aforementioned cell counts (data not shown).

Gender Is the Main Source of Variation in the Neurogenesis-Related Cell Counts

The first principal component (PC1) accounted for 28% of the variation in the cell count data (Ki67, DCX, and cell death) and was driven by sex differences [$t(24) = 2.19$, $P = 0.038$]. This can be seen in Figure 3, in which the data are slightly separated on the *x*-axis (PC1) when grouped by male versus female. The second and third PCs were not related to genotype or age.

Learning Abilities in Males and Females Senescent Mice

On introduction in the novel environment, female subjects appeared more explorative than males visiting more often all the corners during the first 3 h [sex by time interaction: $F(2,50) = 4.094$; $P = 0.0226$, post hoc comparisons females-3 h vs. males-3 h, $P < 0.01$; Fig. 4A]. Interestingly, after 24 h, KO mice, regardless of sex, showed an increase in their explorative profile that was subsequently lost [genotype by days interaction: $F(1,25) = 3.404$; $P = 0.0411$; post hoc comparisons: KO-day 1 vs. WT-day 1, $P < 0.01$; Fig. 4B]. Overall, KO subjects did not differ from WTs as for their cognitive abilities [main effect of genotype: $F(1,25) = 0.221$; $P = 0.6423$ for place-learning task and $F(1,25) = 0.020$; $P = 0.8899$ for reversal task]. However, a main effect of gender showed that old females were characterized by a superior

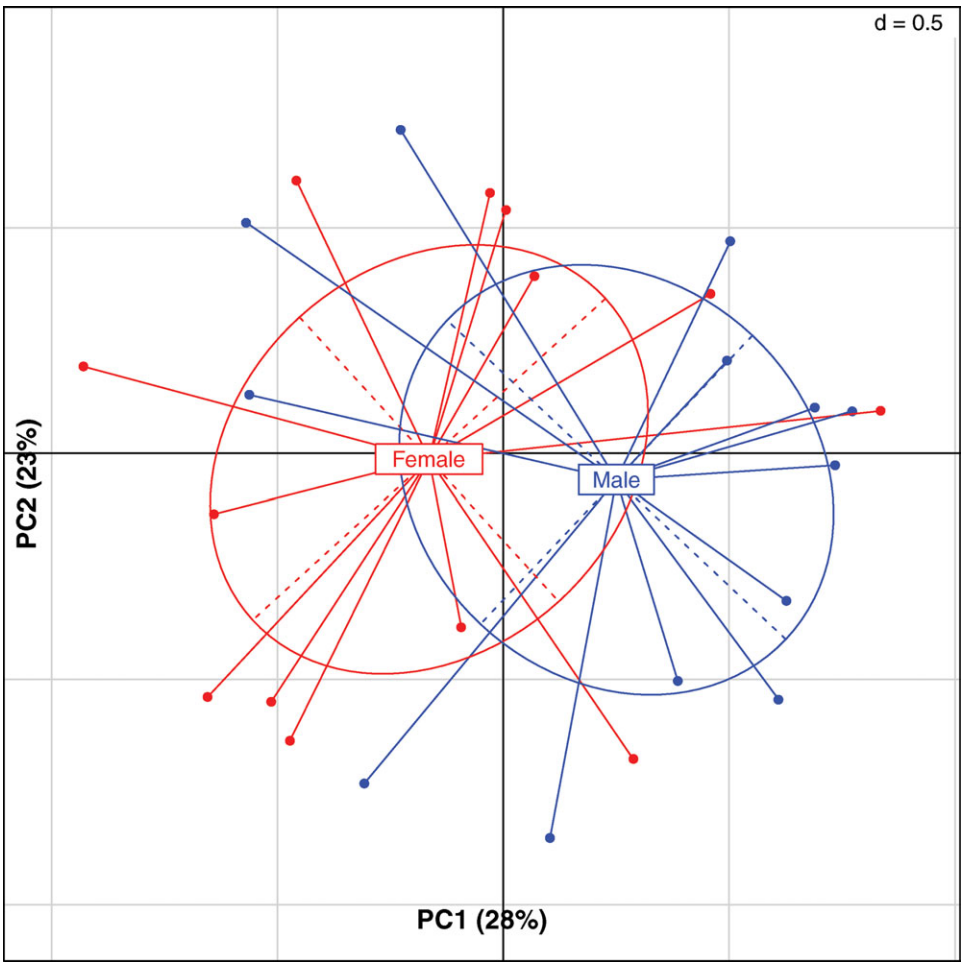

FIGURE 3. First principal component (PC1) is driven by sex differences [$t(24) = 2.19$, $P = 0.038$] and accounts for 28% of the variation in the cell count data (number of proliferating cells, young cells of the neuronal lineage, and dying cells). The second principal component (PC2) could not be related to a given factor such as genotype or age. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

learning performance during the task (lower percent of errors in spatial learning) in comparison with male mice [$F(1,25) = 7.567$; $P = 0.0109$; Fig. 5A]. This difference did not apply to the reversal phase [$F(2,50) = 0.331$; $P = 0.7196$; Fig. 5B].

DISCUSSION

Unexpectedly, we found that the main effect on morphological, behavioral, and neurogenesis-related variation in senescent $p66^{Shc-/-}$ and wild-type mice is driven by sex. Females are less affected by age-dependent decreases in brain weight and neurogenesis, retain exploration activity, and show better performance in place-learning task than males. Depletion of $p66^{Shc}$ is associated with a higher proliferation in females, who may be protected from precursor cell loss. Our data suggest that hippocampal function is protected in females, an effect that is amplified in the $p66^{Shc-/-}$ mutants.

Senescent Females Show Increased Numbers of Young Neurons

Several lines of evidence suggest a gender-specific regulation of adult hippocampal neurogenesis related to steroid hormones (for review, see Galea et al., 2008; Pawluski et al., 2009). Depending on strain and age, proliferation and/or survival of young neurons differ between female and male rats (Perfilieva et al., 2001; Falconer and Galea, 2003). The level of glucocorticoids, known to increase while aging, reduces neurogenesis in old rats (Cameron and McKay, 1999). However, direct comparisons of female and male mice within the same study are rare. In young adult (6–7 weeks) C57BL/6 mice, neurogenesis is not influenced by gender or estrogen cycle (Lagace et al., 2007). However, at the age of 23 weeks, C57BL/6 males have been reported to have more young neurons than females (Clark et al., 2008). In contrast, at the age of 25 weeks, but not at younger ages, female CBA mice have more DCX-positive cells than males (Lazic et al., 2006). Hippocampal neurogenesis can

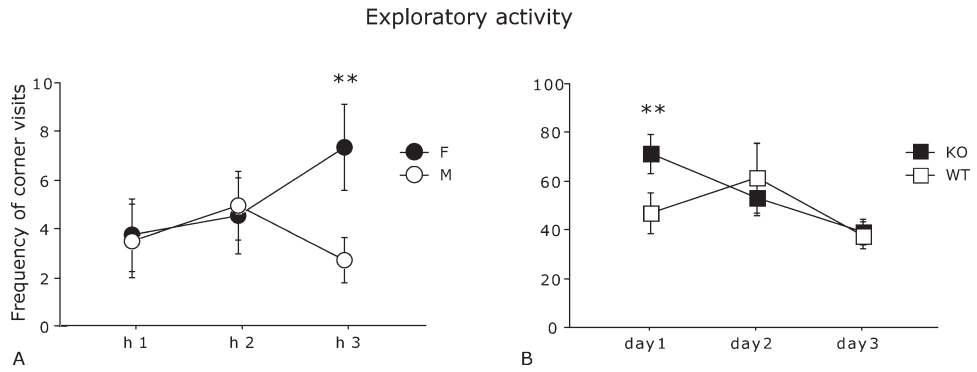

FIGURE 4. Exploratory activity in the IntelliCage. Overall, female subjects, on introduction in the novel environment, appear more explorative than males visiting more often all the corners, where they have access to water, during the first 3 h (A). After 24 h, KO mice, regardless of sex, showed an increase in their explorative profile that was successively lost in the following 2 days (B). Data are represented as mean ± SEM (F, females, $n = 15$; M, males, $n = 14$; WT: $p66^{Shc+/+}$, $n = 14$; KO: $p66^{Shc-/-}$, $n = 15$); $^{**}P < 0.01$ both for F-3 h vs. M-3 h (A) and for KO-Day 1 vs. WT-Day 1 (B).

be modulated by environmental stimuli such as OS, which could act as an inhibitory factor especially on proliferation of neural progenitor cells (Verity, 1994). Our findings of more young cells of the neuronal lineage in old $p66^{Shc-/-}$ females when compared with $p66^{Shc-/-}$ males and an overall higher number of young neurons in senescent females when compared with males emphasize gender-specific differences that might become apparent only at older ages.

Sustained Pool of Precursor Cells in the Hippocampus of $p66^{Shc-/-}$ Female Mice

In the $p66^{Shc-/-}$ mice, we observed increased cell proliferation specifically in females. One of the characteristics of the $p66^{Shc}$ knock out is a vascular resistance against OS (Napoli et al., 2003; Camici et al., 2007), an effect that is particularly apparent while aging (Francia et al., 2004). The vascular niche, that is, a close association of the vasculature with dividing precursor cells, is thought to provide a favorable environment for these cells (Palmer et al., 2000). A disruption of the vascular niche during aging has been discussed as one of the factors leading to the age-dependent downregulation of hippocampal cell proliferation (Hattiangady and Shetty, 2008). $P66^{Shc-/-}$ mice are less affected by the negative impact of angiotensin II on cardiovascular cells (Graiani et al., 2005), an effect that might be potentiated in female KO as female mice of other strains have been shown to harbor more angiotensin II receptor type 2 than males (Sakata et al., 2009). Another factor that could explain the marked difference in proliferation between WT and KO females is that $p66^{Shc-/-}$ females become reproductively active at a younger age; however, the rate of successful births also decreases earlier than in wild-type females (Giorgio et al., 2012). Hormonal changes in $p66^{Shc-/-}$ females, entering menopause earlier than normal, could have an effect on precursor survival as well. In addition, KO females might benefit more from lack of $p66^{Shc}$ gene than males by way of synergistic

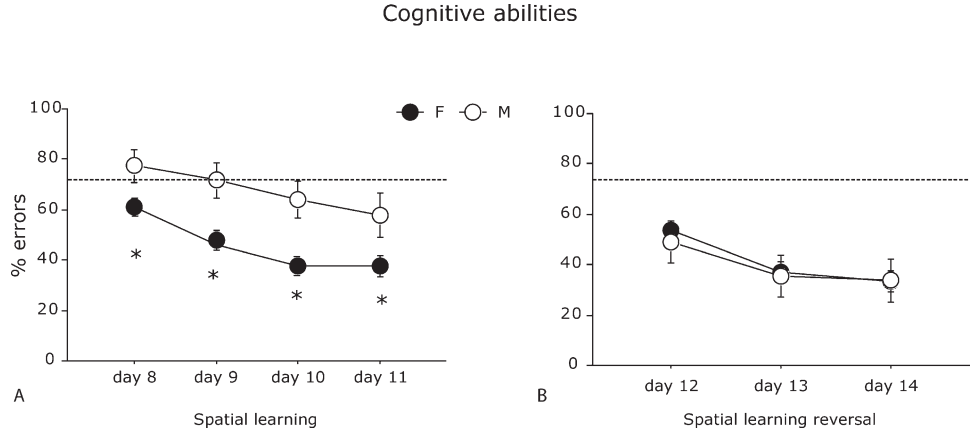

FIGURE 5. Cognitive abilities in the IntelliCage. During the spatial learning phase, female subjects showed a learning curve characterized by lower errors than males (A); this did not apply to the spatial learning reversal phase, where males and females appeared to be characterized by comparable behavioral plasticity (B). Dashed lines represent chance level (75%). Data are represented as mean ± SEM (F, females, $n = 15$; M, males, $n = 14$); $^{*}P < 0.05$.

effects of reduced OS and estrogen, a mechanism which has been found to exert a neuroprotective effect during aging (Bruce-Keller et al., 2000; De Nicola et al., 2009).

Less Granule Cells in Aged $p66^{Shc-/-}$ Mice Is Likely a Remainder of Developmental Processes

Previous studies do not report obvious macroscopic neuroanatomical alterations in KO mice, indicating that only the lack of the p46 and/or p52 isoforms lead to microcephaly, but not ablation of $p66^{Shc}$ (Migliaccio et al., 1999; McFarland et al., 2006). Although not comparable with the microcephaly induced by other isoforms, the reduction in brain size found in the $p66^{Shc}$ KO mice here supports a neurodevelopmental defect, suggesting that $p66^{Shc}$ KO effects extend beyond the hippocampus. $p66^{Shc}$ proteins are expressed embryonically in the proliferating neuroepithelium (Conti et al., 1997) that has been described as one of the embryonic sources of cells migrating into the hippocampus to form the granule cell layer in rats (Altman and Bayer, 1990). A lack of the $p66^{Shc}$ protein during development therefore interferes with hippocampal development in $p66^{Shc-/-}$ mice, which is detectable as modestly lower numbers of granule cells as adults. However, it should be noted that our estimates of total granule cells in senescent mice with background strain 129Sv/Ev, both in wild type (5.3×10^5) and $p66^{Shc-/-}$ (4.8×10^5), are considerably higher than in 9-week-old 129Sv/J mice (mean = 2.8×10^5; Kempermann et al., 1997). The few studies where total granule cell number has been evaluated in different age categories do not indicate that ongoing neurogenesis augments total granule cell number (Ben Abdallah et al., 2010, and references therein), suggesting that the reduced number of granule cells in KO mice is a consequence of early developmental processes. Our finding that the volume of the mossy fiber terminal fields, the output structure of granule cells, does not differ between genotypes is rather puzzling. Mouse strains differ significantly in the total number of granule cells (Kempermann et al., 1997) and mossy fiber volume (Crusio et al., 1993). To our knowledge, there is no other study that assessed both parameters. When we analyzed data of 11 BXD recombinant mouse strains for total granule cells (Kempermann and Gage, 2002) and mossy fibers (Lassalle et al., 1999), we could not find a correlation between the two measurements. It appears that the size of the mossy fiber terminal fields is differently regulated and independent of $p66^{Shc}$.

Increased Exploration in $p66^{Shc-/-}$ Mice and Better Learning Abilities in Female Subjects

Overall, old KO mice were more ready to explore the environment upon introduction in IntelliCages. Increased locomotor and exploratory activities were already observed in adult KO male mice (Berry et al., 2007, 2008). Here, we show that this behavioral phenotype is maintained not only in senescent males but also in female subjects. We have previously shown that increased exploration in the mutants is most likely linked to the metabolic needs and thus could be interpreted as "food-seeking" behavior (Giorgio et al., 2012).

On introduction in the IntelliCage, all females showed a prompt exploration of the environment, an effect which vanished with time. One week later, all subjects were tested for spatial learning abilities (Krackow et al., 2010). We have previously shown that both WT and KO senescent mice are poor learners in the MWM as this test relies heavily on motor abilities and is very stressful for old subjects (Berry et al., 2008). Thus, this time, we selected a spatial task more suited for old subjects and tested for the first time old females. No differences emerged between genotypes. Surprisingly, however, all females made fewer errors in the place-learning task, showing a persistent greater preference for the rewarded place than males. Both males and females performed alike in the reversal phase, indicating efficient replacement of a former spatial preference in senescent mice of both genders. The initial better discrimination for correct choice in females can be interpreted as faster behavioral adaptation relative to males.

CONCLUSION

The physiological age-related memory loss, and the often associated neurodegeneration, is a slow but progressive process, beginning early during aging in animals, including humans, which strongly impacts the quality of life. The generation of ROS and/or free radical-induced OS, which is the major age-related change, can lead to hippocampus damage and increase vulnerability to impaired learning and memory. Our data showing higher cell proliferation in $p66^{Shc-/-}$ female mice might suggest that reduced OS, directly or indirectly, could cooperate to protect hippocampal function in the mutants. This study also shows that senescent female mice in general are less affected by age-related changes. Behavioral and morphological data are derived from different animals, and thus, causal model statistics cannot be applied (Lazic, 2012). However, our findings will facilitate future studies investigating gender effects in senescence, an effect that is well described in humans (Kryspin-Exner et al., 2011) but has received little attention in animal models.

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