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Regenerative capacity of neural precursors in the adult mammalian brain is under the control of p53

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Abstract

The question of whether or not stem cell loss drives aging in the brain is not fully resolved. Here, we used mice over-expressing the short isoform of p53 (Δ Np53 or p44) as a model of aging to gain insight into the cellular mechanisms underlying age-related brain functional deficits. By BrdU labeling, we observed an accelerated decline in the number of subventricular zone proliferating cells with age in p44Tg mice compared to mice with normal p53 expression. A 2–3-fold reduction in the number of slowly dividing stem cells was evident in the subventricular zone of 9–12 month-old p44Tg mice, but not in younger p44Tg mice or in normal mice. Consequently, the supply of new olfactory bulb neurons was also reduced. The number and size of neurospheres generated from subventricular zone cells from p44Tg mice was significantly reduced, and cells derived from these neurospheres had limited self-renewal and amplification capacities. At the cellular level, p44 lengthened the cell-cycle and affected cell cycle reentry properties, evident by an increased proportion of cells in G0. At the functional level, p44 expression resulted in impaired olfactory discrimination in 15–16-month-old mice. This phenotype is driven by constitutive activation of p53 and constitutive expression of p21^{Cip1/waf1} in neural stem cells. Our results demonstrate that p53 plays a crucial role in the maintenance of the regenerative capacity of the brain by regulating the proliferation of stem and progenitor cells.

Keywords

Δ Np53; p44; aging; neural stem cells; neurospheres; subventricular zone; olfactory bulb; olfaction

1. Introduction

In mammals, neurogenesis continues throughout life in two regions of the adult brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus [2,11]. Stem cells in the SVZ generate neural precursors that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into granule cells and periglomerular interneurons [11,16,38]. Stem cells in the SGZ generate intermediate

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Disclosure Statement

The authors declare that there are no actual or potential conflicts of interest.

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precursors that differentiate into granule cells in the dentate gyrus [2,7,24,57]. The integration of newly generated neurons from stem cells in the SVZ and SGZ is important for maintaining odor discrimination [25], and for learning and memory [5,27,58,59], respectively, functions necessary for survival in several if not most mammalian species.

The gradual loss of sensory and cognitive functions that occurs as healthy adults age is paralleled by a decline in adult neurogenesis [23,28,33,35,56,64]. In the SVZ, there is an age-dependent loss of stem cells [40], but whether or not stem cell loss actually drives aging and functional impairment in the brain or elsewhere is not known. One possibility is that the loss of stem cells is due to altered cell division, as suggested by experiments using mice deficient in the cell cycle regulator p21^{Cip1/Waf1} (p21). Neural stem cells deficient in p21 have been shown to undergo rapid cell division due to their inability to maintain quiescence, and prematurely exhaust their ability to maintain neurogenesis [34]. Loss of the tumor suppressor p53, the upstream activator of p21, has the same effect [26,42]. These results suggest that the length of time neurogenesis can be maintained in the adult might be dependent on the capacity of stem cells in neurogenic regions of the brain to proliferate, and that this is controlled at least in part by p21 and p53.

The activity of p53 depends on the relative amounts of at least two isoforms of the protein, the full-length form and a truncated, more stable form lacking the N-terminus [12,68]. The ΔNp53 isoform, which is missing the first 40 amino acids of the protein, is 44 and 47 kD in size in mouse and human, respectively [12,53]. Changes in the pattern of p53 isoform expression that cause constitutive activation of p53 are associated with a decreased risk of tumorigenesis and premature aging in genetically altered mice [39,65]. Increased dosage of ΔNp53 results in increased transactivation of the p21 gene and a concomitant reduction in cell proliferation [39]. If increased ΔNp53 affects the proliferation of stem and progenitor cells in the brain as well, then localized failures in neurogenesis could translate into the type of cognitive and/or sensory deficits commonly seen in old age.

We have used mice with an increased dose of ΔNp53 (p44Tg mice) to ask questions about the role of stem cell proliferation in aging. If the loss of neural stem cells is due to altered cell division, then stem cell proliferation should be slower than normal in these mice. If the length of time neurogenesis can be maintained in the adult is dependent on neural stem cell division, then neurogenesis should be maintained for a shorter time in these mice. Finally, if stem cell loss actually drives brain aging (*i.e.*, the loss of brain function), then function should decline in parallel with loss of stem cell proliferation. To test these hypotheses, we examined the proliferation of neural stem and progenitor cells (NSCs) *in vivo* and *in vitro*. We determined that the impaired ability of NSCs to proliferate does indeed limit the supply of newly generated neurons in the adult brain in an age-dependent way. We also determined that brain function (olfaction) and stem and progenitor cell proliferation declined in parallel. We propose that p53 is a central regulator of neurogenesis in the adult mammalian brain. p53 activity can be modulated by altering the relative amounts of transactivation-competent subunits (p53 monomers) and transactivation-incompetent regulatory subunits (p44 monomers) that make up functional p53 tetramers. Our results suggest that during aging this regulatory mechanism deteriorates, resulting in disruptions in the ability of stem cells to proliferate. As a consequence, neurogenic regions in the adult brain lose the capacity to replace neurons lost through attrition and normal brain function declines.

2. Methods

2.1. Materials

All reagents were of reagent grade available from commercial sources.

2.2. Animals

The *p44* mutation consists of a 3 kb deletion within the *p53* gene that removes exon 2 and the flanking parts of introns 1 and 2 [45]. Translation initiates at Met 41 in exon 4 producing a 44 kD protein [53]. Transgenic mice carrying the truncated form of *p53* were initially generated by microinjecting a genomic fragment containing the *p44* mutation (a gift from Alan Bernstein; [36]) into B6SJL embryos. The transgene was transferred to an ICR background and bred to homozygosity generating the P+/+ and Q+/+ lines used in this study. Mice were housed under standard conditions with four animals per cage and kept on a 12 h-light/12 h dark cycle at 22 °C and 50–60 % humidity. Food and water were given *ad libitum*. All *in vivo* experiments were conducted using aged-matched male transgenic (P+/+), and non-transgenic (NT) mice. For NS cultures, NT, P+/+ and Q+/+ female mice were used.

2.3 Bromodeoxyuridine injections

Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was dissolved in 0.9% saline containing 0.007 M NaOH at a concentration of 5 mg/ml. To label proliferating cells in the brain, mice received two injections of BrdU (100 mg/kg; i.p.) 24 h apart and were killed 2 h after the second injection. For long-term analysis of labeled SVZ dividing cells, BrdU (100 mg/kg; i.p.) was injected twice a day for two days and mice were killed 12 days after the last injection. It is accepted that cells that remain labeled in the SVZ for at least a week after BrdU or [³H] thymidine treatment are potential slowly dividing, stem cells [8,10,17,32,44]. This interval is sufficient to allow newly generated cells to undergo migration, differentiation or apoptosis.

2.4. Tissue processing and staining

Animals were killed by pentobarbital overdose. The brains were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C for 24 h and cryoprotected with 30% sucrose. Serial frozen coronal sections through the OB, the RMS and SVZ were cut at 25 µm thickness using a Leica CM1850 cryostat (Leica Microsystems, Nussloch, Germany). For histological analysis, sections were stained with 0.1% cresyl violet. For BrdU, PSA-NCAM and GFAP immunostaining, mounted sections were immersed in methanol at –20 °C for 10 min and microwaved in 10 mM sodium acetate for 20 min for antigen unmasking [9]. Sections were blocked with 10% normal goat serum (NGS), 3% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS at room temperature (RT) for 1 h. Sections were then incubated at 4 °C for 24–48 h with the following antibodies diluted in PBS supplemented with 10% NGS and 3% BSA: anti-BrdU (mouse monoclonal, 1:1000; developed by S.J. Kaufman; obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242); anti-GFAP (rabbit polyclonal, 1:200; Dako, Carpinteria, CA); anti PSA-NCAM (mouse monoclonal, 1:100; Chemicon, Temecula, CA); anti-DII/Dlx2 (rabbit polyclonal; 1:100, kindly provided by Grace Boekhoff-Falk, University of Wisconsin). Sections were incubated with Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-mouse IgM (1:300; Molecular Probes, Eugene, OR) at RT for 1 h and counterstained with DAPI 5 µg/ml (Molecular Probes) or 10 µM DRAQ5 (Biostatus Limited, Leicestershire, UK). For BrdU and NeuN double immunostaining, OB floating sections were incubated in 50% formamide, 2x SSC at 65 °C for 2 h and then treated with 2N HCl at 37 °C for 30 min. After being incubated in 0.1M Na borate at RT for 30 min, sections were blocked as described above, and incubated at 4 °C for 48 h with anti-BrdU (rat monoclonal, 1:50; Abcam, Cambridge, MA) and anti-NeuN (mouse monoclonal, 1:100; Chemicon, Temecula, CA). Sections were incubated with Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 586 goat anti-mouse IgG (1:300; Molecular Probes, Eugene, OR) at RT for 1 h and counterstained with DAPI.

2.5. Neurosphere culture

Neurospheres (NS) were generated from the SVZ of adult NT, P+/, and Q+/+ mice as described [8] and cultured under clonal conditions according to published procedures [34]. Briefly, for each culture the entire SVZ region was dissected from sagittally cut brains, incubated in DMEM medium containing 20 U/ml papain (Worthington, Lakewood, NJ), 1mM *N*-Acetyl-L-cysteine (NAC; Sigma, St. Louis, MO) and 2 U/ml RQ1 DNAase (Promega, Madison, WI) for 1 h at 37 °C. Dissociated cells were plated in 24-well plates at a density of 2.6×10^3 cells/cm² (5×10^3 cells in 0.5 ml/well) in DMEM containing N2 (Invitrogen, Carlsbad, CA), B-27 (StemCell Technologies, Vancouver, Canada), 1 mM NAC, 20 ng/ml EGF and 20 ng/ml bFGF (both growth factors recombinant human from Invitrogen). It has been reported that under these culture conditions, NS are generated from single cells [34]. The total number of NS at P0 was determined at 7 days *in vitro* (7 DIV) by counting all NS generated from each mouse brain. In subsequent passages, the total number of NS was determined by counting all the NS generated in 12 wells of a 24-well dish seeded at 5×10^3 cells/well and extrapolating for the total number of dissociated cells. Self-renewal was determined as the number of secondary NS at 7 DIV/number of seeded cells $\times$ 100. Amplification was calculated as the number of viable cells from dissociated NS at 7 DIV/number of seeded cells. NS were counted at 40X magnification using a grid objective and phase contrast microscopy. The number of viable cells was determined in samples of SVZ- or NS-dissociated cells by trypan blue exclusion using a hemocytometer. NS diameter was measured in phase contrast pictures of individual NS using the ImageJ software (Wayne Rasband; National Institutes of Health; USA).

2.6. Immunocytochemistry

NS were collected at 7 DIV and allowed to attach to poly-L-ornithine coated glass slides in growth medium for 30 min at RT. NS were fixed with 4 % PFA at RT for 10 min and in 4 % PFA plus 0.1 % Triton X-100 at 37 °C for 10 min., incubated in 0.2 N HCl at RT for 10 min, 5 mM MgCl₂, 0.1% Triton X-100 in PBS at RT for 5 min, and blocked with 10 % NGS, 0.1 % Triton X-100 in PBS for 1 h at RT. NS were then incubated at 4 °C for 24 h with the following antibodies diluted in PBS supplemented with 10% NGS: anti-phospho-p53 (Ser15) (rabbit polyclonal, 1:100; Cell Signaling Technology, Danvers, MA); anti-p21 (mouse monoclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were incubated with Alexa Fluor 488 goat anti-rabbit and anti-mouse IgGs (1:300; Molecular Probes) at RT for 1 h and counterstained with DAPI.

2.7. Apoptosis

Fluorescence in situ end labeling (FISEL) was performed as described [61]. Staining was conducted in 25 μ m-thick floating sections and in isolated cells and NS plated on glass slides and fixed with 4% PFA at RT for 15 min. Sections and cells were incubated with 100 μ l of a reaction mix containing 1 μ l (25 U) terminal transferase (Roche Applied Science, Indianapolis, IN), 20 μ l 5x cacodylate buffer, 10 μ l 25 mM CoCl₂, 8 μ l 0.5 mM dTTP, 1 μ l 1 mM Biotin-16-UTP and 60 μ l ddH₂O at 37 °C for 1 h. Biotin-16-UTP incorporation was revealed with Alexa 488 conjugated streptavidin (Molecular Probes). Sections and cells were counterstained with DAPI. For active caspase-3 staining, PFA-fixed cells or NS were blocked with 10% NGS, 0.1 % Triton X-100 in PBS at RT for 1 h followed by incubation with anti-active caspase-3 (rabbit monoclonal, 1:500; BD Biosciences, San Jose, CA) at 4 °C for 24 h. Staining was revealed with 488 Alexa Fluor goat anti-rabbit IgG (1:300; Molecular Probes). Nuclei were counterstained with DAPI.

2.8. Cell cycle analysis

Acridine orange staining—The percentage of cells in each phase of the cell cycle, G0, G1, S and G2/M, was determined by acridine orange staining as described [1]. Acridine orange intercalates into double-stranded DNA resulting in green fluorescence (maximum emission at 530 nm) and binds to single-stranded RNA resulting in red fluorescence (maximum emission at 640 nm). Since cells in G0 have 5 to 10 times less RNA than cells in G1, acridine orange can differentiate between the two phases of the cell cycle. NSCs were seeded at 1×10^4 cells/well in 24-well plates, grown for 4 days under normal conditions in the presence of EGF and bFGF, and dissociated with papain. Single cells suspended in 80 μ l of growth medium containing 10% serum were permeabilized with 120 μ l of a solution containing 0.1% Triton-X100, 0.1 N HCl and 0.15 N NaCl for 15 sec and stained with 320 μ l 5 μ g/ml acridine orange in 5 mM EDTA, 0.15 N NaCl, 0.1 M phosphate citrate, pH 6.0 for 3 min. Cells were counted using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). At least 4,000 events per sample were analyzed using FlowJo software.

Cumulative BrdU staining—To determine the length of the cell cycle and the length of the S-phase of NSCs, cells were seeded at 1×10^4 cells/well in 24-well plates and grown under normal conditions in the presence of EGF and bFGF. Four days after plating, cells were pulsed with 1 μ M BrdU for the last 1, 3, 6, or 12 hours of culturing, harvested, and dissociated with papain. Cells were placed onto poly-L-ornithine-treated microscope slides in growth media for 1 h, fixed with 4% PFA in PBS for 20 min at RT, treated with 2 N HCl at RT for 30 min followed by 0.1M sodium borate, pH 8.5 for 30 min. Cells were blocked with 10% NGS, 3% BSA, 0.2% Triton X-100 in PBS at RT for 1 h and then incubated at 4 °C for 24–48 h with anti-BrdU (mouse monoclonal, 1:500). Cells were incubated with fluorescently labeled secondary antibodies at RT for 1 h and counterstained with DAPI. BrdU positive cells were counted using a Zeiss Axiophot fluorescence microscope and expressed as a percentage of total number of cells determined by DAPI nuclear staining. Regression analysis of the linear portion of each BrdU labeling curve was used to estimate total cell cycle and S-phase times as described [47,48].

2.9. Microscopy

Tissue sections and cultured cells were visualized using a Zeiss Axiophot fluorescence microscope. Fluorescence images were captured using a Sony DSC-S7 digital camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA). Confocal images were obtained using a standard Zeiss LSM 510 confocal microscope equipped with an argon/2 488 nm, and HeNe 543 nm and 633 nm lasers. For visualization of BrdU and GFAP double staining in lateral ventricle sections, Z stack images were captured at 1- μ m intervals with a 40X objective and a pinhole of 1.0 Airy unit. Immunostaining for BrdU and GFAP was considered to be in close juxtaposition, in cells exhibiting green stained nuclei (BrdU) entirely surrounded by red fluorescence (GFAP) at a distance smaller than 0.5 μ m. For p21 and phospho-p53 (Ser15) immunostained neurospheres, Z stack images were captured at 1- μ m intervals with a 40X objective and a pinhole of 1.0 Airy unit. To reduce signal to noise ratio, each optical slice was scanned 4 times and the values averaged. The dynamic range of acquisition was optimized using the range indicator in the color palette tool. To allow semi-quantitative comparisons of fluorescence levels between NS, all images were acquired using the same microscope settings. To determine the mean pixel intensity of background staining, we performed negative controls in which the primary antibody was omitted. Image analysis was performed using the LSM 510 Software package (Version 2.8) according to published procedures [30]. The histogram function of the program was used to obtain semi-quantitative data for fluorescence intensity comparison. The middle section of each stack was quantitated. The mean fluorescence intensity in arbitrary units was determined for each image after subtracting background levels using the low threshold tool.

2.10. Cell counting and statistical analysis

At least three mice of each genotype were analyzed. The number of BrdU positive cells in the anterior portion of the lateral ventricle (level C in Figure 1 A, Bregma 1.10 mm to 0.98 mm) was counted in six consecutive adjacent hemisections. In experiments involving long-term analysis of BrdU-labeled cells in the SVZ, BrdU positive cells were counted in one every four non-adjacent hemisections throughout the lateral ventricle (Bregma.1.18 mm to 0.26 mm). All cell counts were performed using a Zeiss Axiophot fluorescence microscope at 40X magnification. Areas occupied by the SVZ of the lateral ventricle were measured in DAPI stained sections using Image-Pro Plus image analysis software (Media Cybernetics Inc., Carlsbad, CA).

The number of FISEL positive cells was estimated in 1 out of 4 non-adjacent 25 μ m coronal olfactory bulb sections from the anterior portion of the bulb to the beginning of the accessory olfactory bulb. The density of FISEL positive cells was determined in the granule cell layer at 40X magnification within a defined grid area (250 $\times$ 250 μ m in the dorsal and ventral regions, and 200 $\times$ 250 μ m in the medial region).

To estimate the density of cells in different layers of the OB, the number of Nissl-stained cell bodies was determined at 40X magnification within a defined grid area (50 $\times$ 250 μ m in the mitral/tufted cell layer, medial side; 125 $\times$ 250 μ m in the glomerular layer, lateral side; 100 $\times$ 150 μ m in the granule cell layer, medial side). Three representative 25- μ m coronal sections through the main OB from each animal were analyzed. Each area was counted three times and the mean calculated.

Results are expressed as means $\pm$ standard deviation. Statistical comparisons between groups were performed using two-way ANOVA followed by post hoc comparison using Tukey HSD test, or by two-tailed Student's *t* test.

2.11. Olfactory Discrimination Procedure

Animals—Four male P+/+ mice and 5 male NT mice bred in our colony were assessed for olfactory sensitivity at 15–16 months of age. The subjects were housed individually on a 12 h light:dark cycle (lights on at 06:00). Food and water were available *ad libitum* unless otherwise stated.

Training—Training and testing took place in the home cage between 10:00–14:00 during the light phase of the photocycle. Mice were water deprived for 18 h prior to each session. Subjects were initially trained to drink 10 μ l of almond scented water (made with almond extract, Nielsen-Massey Vanillas, Waukegan, IL) from a clear plastic dish (100 $\times$ 15 mm) placed in the home cage. After 2–3 days of *ad libitum* water, the subjects were water deprived for 18 h and discrimination training began. During discrimination training, each subject received 5 2-min trials in which 5 μ l of 40% almond water was added to 5 μ l of water. This was followed by 5 2-min trials in which 5 μ l of 40% coconut water (made with coconut extract - McCormick & Co, Hunt Valley, MD) was added to 5 μ l of a 0.5% denatonium benzoate (DB) (Sigma) solution (0.5 g DB dissolved in 10 ml water). DB is extremely bitter and therefore is very aversive to mice. Consumption of the DB solution resulted in vigorous head shaking and rubbing the muzzle in the bedding. Rodents easily learn to avoid an odor that has been paired with an aversive event [20].

Testing—Testing began immediately after discrimination training was completed. The mice were first tested for their ability to discriminate between almond and coconut odors and to show a preference for almond. During each of the 10 tests, subjects were presented with 10 μ l of solution in 2 plastic dishes. One stimulus consisted of 5 μ l of water added to 5 μ l of 40%

almond water. The other stimulus was 5 μ l of 40% coconut water added to 5 μ l of 0.5% DB. A test began when the dishes were placed at opposite ends of the subject's home cage and ended after the subject had investigated both stimuli and drank at least one. A subject was scored as making a correct response if it consumed the almond stimulus and did not consume the coconut stimulus. An incorrect response was recorded if the subject drank any of the coconut stimulus, either before or after drinking the almond stimulus. With this scoring system, the range of possible scores for each subject was 0 (all 10 trials incorrect) to 10 (all 10 trials correct). The average inter-trial interval (ITI) was 30 sec and the stimulus location was counterbalanced. This set of tests was designated as 100:0, since each solution consisted of only one odor, almond or coconut.

Subjects were then tested on a more difficult task in which they had to discriminate between a stimulus that was mostly almond (with some coconut) and another stimulus that was mostly coconut (with some almond) in order to avoid consuming the DB. This procedure has previously been demonstrated as an effective means of assessing olfactory sensitivity [23]. Execution and scoring of these tests were identical to that described above with the exception that one stimulus consisted of 5 μ l of water added to 5 μ l of a solution that was 60% almond water and 40% coconut water and the other stimulus consisted of 5 μ l of a 60:40 coconut:almond water solution added to 5 μ l 0.5% DB. Each subject received 10 tests with an average ITI of 30 sec. This set of tests was designated as 60:40. The length time elapsed from the beginning of discrimination training to the end of testing ranged from 2–3 hrs.

Number of correct responses was analyzed using a 2-way ANOVA with genotype (P+/+ versus NT) as a between group variable and stimulus ratio (100:0 and 60:40) as a within group variable.

3. Results

3a. Increased Δ Np53 (p44) impairs adult neurogenesis

Reduced adult neurogenesis is a hallmark of aging in mammals. We have previously reported that over-expression of the short form of p53, p44, in mice causes an accelerated loss of health and life that resembles what we call premature aging [39]. To determine if the premature aging phenotype of p44Tg mice was accompanied by reduced neurogenesis, we studied cell proliferation in the SVZ of the lateral ventricles of brains from non-transgenic (NT) and p44 transgenic (P+/+) mice by BrdU incorporation. BrdU, a thymidine analog, is incorporated into DNA during S-phase of the cell cycle and can be detected immunohistochemically in cell progeny [15,47]. Figure 1B shows BrdU immunostaining along the walls of the lateral ventricles of 2- and 10-month-old NT and p44Tg mice. BrdU positive cells were present throughout the lateral ventricle subependyma in young animals of both genotypes, but were more sparsely distributed in older transgenic mice compared to NT mice. Quantitative analysis, performed by calculating the density of BrdU positive cells in the SVZ, showed a pronounced decrease in NSC proliferation with age in p44Tg mice (Fig. 1C). ANOVA analysis revealed that cell proliferation was significantly reduced at 6–7 months of age in transgenic mice, with a 56% reduction in the density of BrdU positive cells compared to young transgenic mice. In 9- to 12-month-old p44Tg mice, cell proliferation was further reduced to 11% of young NT mice. On the other hand, differences in cell proliferation among these age groups were not significant in NT mice. In addition, while there were no significant differences in the number of BrdU positive cells per unit area between NT and p44Tg young mice, cell proliferation was significantly different between the two genotypes at 6–7 months and 9–12 months of age. These results indicate that increased p44 results in a decline in cell proliferation in the lateral ventricle subependyma similar to that normally occurring in much older mice [23,40,64].

The SVZ of the lateral ventricle is composed of several types of proliferating cells, including astrocytes (Type B cells), transient amplifying precursor cells (Type C cells), neuroblasts (Type

A cells), and ependymal cells [18]. Of these, the slowly dividing Type B cells expressing glial fibrillar acidic protein (GFAP), a marker expressed by SVZ astrocytes [17,18], are thought to be the neural stem cells [17,19]. To determine if the population of these slowly dividing GFAP-positive cells in the SVZ of p44Tg mice was altered, we performed proliferation studies in which mice were administered BrdU four times over a period of 48 hours and killed 12 days later. This time interval allowed newly generated cells to undergo division, migration, differentiation or apoptosis and lose the BrdU label. Cells that remained labeled in the SVZ for at least a week after BrdU treatment were considered to be the slowly dividing stem cells, as previously defined [8,10,17,32,44]. Quantitation revealed a 64% reduction in the number of slowly dividing cells in the SVZ of 9–12 month old p44Tg mice compared to young mice. We found no significant differences between the same age groups in NT mice (Fig. 2A). In addition, the number of BrdU positive cells was significantly different between p44Tg and NT mice at 9–12 months but not at 2–4 months. To further characterize these BrdU-retaining cells, we performed double immunostaining for BrdU and GFAP. Confocal microscopy revealed abundant GFAP staining (red), in the processes of SVZ cells and intense nuclear staining (green) of cells undergoing DNA synthesis (Fig. 2B). Juxtaposition of red and green fluorescence in the same optical section was apparent in approximately 88% of the cells analyzed, indicating that the stained structures were localized to the same cell. The majority of BrdU positive cells were negative for *Dlx2*, a marker of C and A cells (Fig. 2C). These results indicate that the stem cell population is reduced in the SVZ of p44Tg mice.

Next, we tested the idea that reductions in NSC proliferation would be reflected in reduced numbers of migrating neuroblasts in the RMS. We examined the population of migrating neuroblasts in the RMS of 10 month-old brain from p44Tg and NT mice by immunostaining with an antibody against the polysialylated form of neural cell adhesion molecule (PSA-NCAM). PSA-NCAM is expressed by neuronal precursors in the adult brain [52] and is essential for their migration from the lateral ventricle subependyma to the olfactory bulb [13]. Coronal sections through the RMS showed that the number of migrating PSA-NCAM positive neuroblasts is dramatically reduced in p44Tg mice compared to NT (Fig. 2D). GFAP immunostaining revealed that, although present, astrocyte elements in the central portion of the RMS are more dispersed in p44Tg brain than in NT brain.

Finally, we sought to determine if the pronounced reductions in proliferating and migrating neuroblasts originating in the SVZ resulted in morphological changes in the target area, the OB. Nissl stained sections from 10-month old p44Tg and NT mice revealed a normal architecture, with all six OB layers (granule cell, internal plexiform, mitral/tufted, external plexiform, glomerular cell, and nerve layers) present (Fig. 3A–B). To determine if reduced SVZ cell proliferation was reflected in a decline in the number of newly generated cells in the OB, we counted the number of BrdU-positive cells in the granule cell layer in mice injected with BrdU 12 days earlier. As shown in Figure 3C, we found a decrease of approximately 50% in the density of labeled cells in dorsal, medial, and ventral areas of the OB in p44Tg mice compared to NT mice. Confocal microscopic analysis of sections immunostained with antibodies against BrdU (green) and the neuronal marker NeuN (red) revealed that ~96% of newly generated cells had differentiated into olfactory bulb neurons. These cells were positive for both markers and thus appear yellow in the photomicrographs shown in Figure 3D. Cell count analysis of Nissl-stained OB sections showed no significant differences in cell density between the two genotypes in young animals in the granule, glomerular and mitral/tufted cell layers. However, we found a significant reduction in cell density in the granule cell and glomerular layers of the OB from old p44Tg animals, but not in the mitral/tufted cell layer (Table I).

3b. Increased $\Delta Np53$ (p44) impairs proliferation of NSCs

To determine if the effect of p44 on stem and progenitor cell proliferation observed *in vivo* is an intrinsic property of the cells, we conducted proliferation studies in isolated SVZ cells grown in culture as NS. The NS assay is a well-established method to study proliferation, self-renewal and maintenance of neural stem cells *in vitro* [44,51]. We found that increased p44 resulted in the generation of NS that were fewer and smaller than NT (Fig. 4A). Size analysis conducted at 7 days in vitro (DIV) showed a significant reduction in the diameter of p44Tg NS, regardless of the age of the donor animal (Fig. 4B, lower panel). Size differences were also apparent in NS generated from Q+/+ mice. Overall, p44Tg NS are 3.6-fold smaller than NT controls. The Q+/+ line has a larger copy number of the p44 transgene than the P+/+ line. Q+/+ mice exhibit a phenotype of accelerated aging but, unlike to P+/+ mice, body size is comparable to NT mice (Fig. 4B, middle panel). In addition, we found a significant effect of genotype and age on the total number of NS generated from NT and p44Tg mice (Fig. 4C). At 2.5 months of age, similar numbers of NS could be derived from transgenic and non-transgenic SVZ. However, at 11.5 months of age, fewer primary NS could be derived from the SVZ of p44Tg mice compared to NT mice. The differences in the number of NS derived from young and old p44Tg mice were also significant. In addition to these reductions in NS size and number, we observed significant decreases in self-renewal (Fig. 4D) and amplification capacity (Fig. 4E) of SVZ cells derived from p44Tg mice compared to NT mice. These differences were present at both 2.5 and 11.5 months of age. These results are consistent with the reduced cell proliferation and neural stem cell number observed *in vivo*. They support the idea that defects in cell proliferation can limit stem cell self-renewal and cause premature stem cell depletion.

p53 is involved in the control of both cell cycle arrest and apoptosis in different tissues including brain. To determine the extent to which apoptosis is responsible for reduced neurogenesis in p44Tg mice, we stained OB sections and NS for apoptosis-specific markers. Active caspase-3 (Fig. 5A upper panel) and FISEL staining (not shown) of intact NS revealed that there were no changes in the number of apoptotic cells in cultures derived from p44Tg mice compared to NT. There was also no difference in the percentage of apoptotic cells in dissociated NS (Fig. 5C). Thus, increased apoptosis cannot account for the impaired production of NS from cells isolated from the SVZ of p44Tg mice. Using similar analysis to detect apoptotic cells in the SVZ of the lateral ventricle and the OB, we did not find any differences between p44Tg and NT mice (Fig. 5D). Quantitation of the number of FISEL positive cells in the granule cell layer of the OB indicated that there were no significant differences in the density of apoptotic cells between transgenic and non-transgenic mice (Fig. 5E). These results demonstrate that impaired neurogenesis in p44Tg mice is not caused by increased apoptosis and support the conclusion that p44 affects the generation of adult neurons primarily by reducing cell proliferation.

p44 over-expression is associated with increased levels of the cyclin-dependent kinase inhibitor and p53 target gene p21 [39]. To determine if p21 levels were elevated in p44Tg SVZ cells, we analyzed NS by confocal microscopy with an antibody against p21. We found that the level of p21 is significantly higher in p44Tg NS compared to NT NS (Fig. 6A). Immunostaining of NS using an antibody against phosphorylated p53 at Ser15 indicated that the activated form of p53 is also significantly increased (Fig. 6B). Altogether, these results indicate that increased p44 interferes with the ability of SVZ stem/progenitor cells to proliferate normally by stabilizing p53 and subsequently increasing p21 levels. Therefore, increases in the steady state level of p53 are accompanied by increased p53 functionality in aging brain.

3c. Increased $\Delta Np53$ (p44) alters the cell cycle of NSCs

The next set of experiments was aimed at gaining insight into the cellular mechanism underlying the effect of p44 on cell proliferation in neurogenic regions of the adult brain. Given the well known role of p53 in cell cycle control, we sought to determine if p44 might affect

cell cycle length and/or reentry properties of neural precursor cells. Using BrdU cumulative labeling and immunocytochemistry, we found that the cell cycle of p44Tg NSCs is 37% longer than that of non-transgenic NSCs (28.3h vs. 20.6h, respectively; Fig. 7A). S-phase was nearly twice as long in p44Tg NSCs compared to NT NSCs (5.8h vs. 3.0h, respectively; Fig. 7A). Next, we analyzed the cell cycle of cells isolated from the SVZ of 3-month-old mice using acridine orange followed by FACS analysis. As reported for embryo-derived NSCs [29], we found that a significant fraction of adult NSCs are in G0/G1, and a small number in G2/M. Overall, we did not find significant differences in the distribution of cells in G0/G1, S, and G2/M stages of the cell cycle between NT and p44Tg NSCs (Fig. 7B, C). However, acridine orange, which binds both DNA and RNA, and distinguishes between cells in G0 and G1, revealed that a larger proportion of the population of cells derived from the SVZ of p44Tg mice are in G0 compared to their NT counterparts (Fig. 7B, C). These results indicate that there are important differences in cell cycle properties between neural precursor cells from normal mice and p44Tg mice, and suggest that impaired G0-G1 cell cycle reentry may contribute to cell proliferation deficits in neurogenic regions of the aging brain.

3d. Diminished brain function is associated with proliferation defects caused by p44

To determine if increased p44 resulted in impaired olfactory function, we conducted olfactory discrimination studies in NT and p44Tg mice. The average number of correct responses each genotype made on both sets of tests is represented in Figure 8. Both genotypes performed well when each discriminative stimulus was composed of only a single odor, either almond, which signaled regular water, or coconut, which signaled bitter water (100:0), responding correctly on nearly all trials. However, when the task was made more challenging by mixing the odors at a 60:40 ratio to create one stimulus that was 60% almond and 40% coconut and another that was 60% coconut and 40% almond, both genotypes performed significantly worse at avoiding the bitter water. This decrement of performance was much more pronounced in the p44Tg group than in the NT group. The analysis revealed a significant main effect of genotype. Overall, p44Tg animals made significantly fewer correct responses than normal (non-transgenic) animals, indicating that olfactory acuity was decreased.

4. Discussion

We have used cells and tissues derived from mice, as well as behavioral studies in mice, to ask questions about the role of stem cells in brain aging. We were specifically interested in the idea that stem cell loss might actually drive the aging process by failing to replace worn out or damaged neurons necessary to maintain key functions. We compared wild-type mice (ICR background) to recombinant mice expressing a transgene derived from a mutant allele of p53 that codes exclusively for the ΔN isoform of p53 (p44Tg mice). Because the transgene is a fragment of genomic DNA and not a cDNA under a heterologous promoter, the product of the transgene is expressed spatially and temporally exactly like the endogenous p53 gene. The net effect is a slight alteration in the ratio between full-length and ΔN p53 isoforms rather than a change in the expression pattern of p53. We described these mice and the consequences of this altered p53:p44 ratio in a previous publication [39], in which we showed that the mice exhibited a progeroid phenotype resembling aging in a number of different tissues. We also showed that proliferation defects in cells derived from transgenic embryos resulted in fewer than normal numbers of cells in differentiated tissues such as the testis and liver. We proposed that this proliferation defect might also impair the ability of stem cells to generate sufficient replacement cells for tissue regeneration, and might underlie the progeroid phenotype of p44Tg mice, and perhaps even normal aging.

We set out to answer three relatively straightforward questions. First, is there a proliferation defect in NSCs in the neurogenic regions of p44Tg adult brain? To answer this directly, we

performed a series of experiments with cells isolated from the SVZ and grown in culture as NS. Differences in the proliferative ability of NSCs isolated from the SVZ of p44Tg mice were evident as early as the third day *in vitro* and became more pronounced as the cultures aged. The average diameter of NS generated from p44Tg mice was significantly smaller than that of NT NS, consistently reaching only about two-thirds the size of their wild-type counterparts within the same period of time. Furthermore, both the self-renewal capability and amplification of cultured NSCs, which are independent measures of proliferative capacity, were impaired in cultures of p44Tg cells. All of these differences point to an intrinsic defect in cell proliferation as being a primary driving force behind the failure of adult neurogenesis in p44Tg mice.

To understand the nature of this intrinsic defect, we compared the cell cycles of NSCs isolated from the SVZ of p44Tg and NT mice. The cell cycle of these cells is known to lengthen during prenatal brain development [6] and in aging mice [64]. We found that NSCs from p44Tg mice have a cell cycle that is 37% longer than the cell cycle of NT cells, and S-phase is twice as long. We think the most significant difference, however, is in the proportion of diploid cells in G0. There are twice as many cells in G0 in the NSCs population in the SVZ of p44Tg mice compared to NT mice. This increased proportion of non-dividing, quiescent cells in neurogenic regions of brains from p44Tg mice, which we now know resemble “older” brains rather than younger brains, provide a simple cell biological mechanism to explain how normal brain function is lost as the organism ages.

The second question we asked is how long adult neurogenesis can be maintained in animals in which NSC proliferation is impaired. The number of NS that can be generated from the SVZ of mice is known to decrease with age. SVZ cells from a normal, 24 month-old animal generated 50%–75% fewer neurospheres than SVZ cells from young adult animals [23,40]. In this study, we show that the number of NS generated from the SVZ of 11.5 month-old p44Tg mice declined even more dramatically, to 3% that of their younger counterparts. This result predicted that, if similar changes had occurred *in vivo*, neurogenesis would decline in p44Tg mice at younger ages compared to non-transgenic mice. By labeling cells with BrdU *in vivo*, we found that the number of proliferating cells in the SVZ of p44Tg mice declined rapidly to 7% for the entire NSC population, and to 35% for the slowly dividing stem cells at 12 months of age. In normal mice, similar but smaller declines have been reported at 24 months of age [23,40,64]. The drop in NSC proliferation in p44Tg brain was accompanied by a reduction in the population of migrating neuroblasts in the RMS. This caused significant differences in the number of newly generated neurons in the granule cell and glomerular cell layers of the OB, where neurons are replaced in the adult, but not the mitral/tufted cell layer, where they are not. Studies conducted in several strains of laboratory mouse, furthermore, indicate that the cumulative effect of continuous neurogenesis is an age-dependent increase in the total number of interneurons in the OB [23,43]. That these interneurons decrease in number in p44Tg mice, therefore, would appear to reflect the combined effect of proliferation defects in all cells leading up to the final differentiation into post-mitotic neurons.

The final question we asked was if impaired neurogenesis affected function. Neurogenesis in the adult OB may be particularly important in rodents, whose reproductive, social, and feeding behaviors depend heavily on olfaction. We identified olfactory deficits in p44Tg mice by comparing the ability of water-deprived mice to discriminate between normal water scented with almond and bitter water scented with coconut. Animals of both genotypes chose the scent associated with normal water (almond) and avoided the scent associated with bitter water (coconut) on nearly every test of the pure stimuli. However, mixing the stimulus odors made the task much more difficult and required greater olfactory sensitivity. Although both groups made more mistakes when discriminating between mixed stimuli, the NT mice were clearly better than p44Tg mice at performing this challenging task. This indicates that the olfactory sensitivity of p44Tg mice was inferior to that of normal mice, and that neuronal function had

declined in parallel with the loss of regenerative capacity of neural precursor cells in SVZ of the adult brain. These results agree with previous studies in normally aging [23] and genetically altered [25] mice indicating that addition of an adequate number of new neurons to the olfactory bulb is important for normal odor discrimination. In humans, there is a decline in both olfactory sensitivity and discrimination with age [14,21,22,63] and this is accompanied by marked morphological changes in the olfactory epithelium and in the olfactory bulb [31,37,41,46,55, 62]. New neurons are generated in the OB of adult humans [4], but currently there is no evidence linking neural precursors in the SVZ to neurogenesis in the bulb [54]. Therefore our results in mice cannot be extended to humans until more is known about the source of stem cells that provide new neurons to the human OB.

A remaining question is whether or not brain aging drives organismal aging. Studies of longevity genes in *C. elegans* have clearly shown that neurons play an important role in life-span regulation [67]. Neuronal expression of p53 appears to suppress longevity in *Drosophila*, with prolongation of life-span achieved by decreasing p53 activity [3]. p44 is a naturally occurring product of the p53 gene [12,68], that can stabilize and alter the activity of p53 [39]. p53 is expressed postnatally in neurogenic areas of the mammalian brain [26,66], and p53-null mice exhibit increased proliferation and self-renewal of SVZ cells [26,42]. Although our results identify p21 and cell cycle arrest as a potential mechanism for the decreased proliferation and self-renewal of SVZ cells in p44Tg mice, other factors may contribute to the p44 effect on neurogenesis. For example, it is possible that neural stem/progenitor cells from p44Tg mice undergo senescence similar to what we have observed in mouse embryo fibroblasts [39]. Another possibility is that increased p44 could have had effects on apoptosis that would alter total cell number. Over-activity of the ΔN isoform of p73, which is similar in structure to the ΔN isoform of p53 encoded by the p44 transgene, is strongly anti-apoptotic in neurons [49,50,60], while loss of p53 has been found to cause an increase in apoptosis [26]. Despite these possibilities, we found no evidence of either pro- or anti-apoptotic effects of p44 on neurogenesis. Our results suggest that increased p44 ($\Delta Np53$) affects an early step in adult neurogenesis that involves the proliferation of stem and progenitor cells rather than a later step that affects the survival of their differentiated progeny. By increasing the activity of p53, p44 accelerates aging and shortens life-span in the mouse [39]. As shown in the present study, it also causes neural stem cell depletion and loss of olfaction. How these two aspects of aging are functionally related will be an important question for further study.

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Abbreviations list

DB	denatonium benzoate
DG	dentate gyrus
FISEL	fluorescence <i>in situ</i> end labeling
GFAP	glial fibrillary acidic protein

NeuN	neuron-specific nuclear protein
NAC	<i>N</i> -Acetyl-L-cysteine
NS	neurosphere
NSCs	neural stem/progenitor cells
NT	non-transgenic mice
OB	olfactory bulb
p44Tg and P^{+/+}	p44 transgenic
PSA-NCAM	polysialylated form of neural cell adhesion molecule
RMS	rostral migratory stream
SGZ	subgranular zone
SVZ	subventricular zone

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Figure 1. Cell proliferation in the subventricular zone of the lateral ventricle of NT and p44Tg mice as a function of age

A. Schematic parasagittal view of the adult rodent brain indicating the regions of neurogenesis and the levels at which cell count analysis and immunostaining for cell-specific markers were performed. **B.** BrdU immunofluorescence in representative coronal sections at the level of the lateral ventricle (level C in panel A) from 2- and 10-month-old NT and p44Tg mice. Green: BrdU. Blue: DAPI. CPu: caudate putamen. Spt: septum. Scale bar: 50 μ m. **C.** Quantitative analysis of BrdU incorporation. Cell proliferation is expressed as the number of BrdU positive cells/mm² and corresponds to counting performed at level C in panel A. Results represent mean $\pm$ S.D. ANOVA revealed a significant main effect of genotype, $F(1,12)=18.12$, $p=0.001$ and

age, $F(2,12)=23.09$, $p<0.0001$. The genotype x age interaction was also significant, $F(2,12)=5.82$, $p=0.0171$. Simple main effect test showed that there was a significant decrease in the number of proliferating cells with age in the p44Tg mice, $F(2,6)=28.17$, $p=0.0009$. Tukey/Kramer test showed significant differences at 6–7 months and 9–12 months compared to 2–4 months, $p<0.05$. The number of BrdU positive cells was not significantly different among the three age groups in NT mice, $F(2,6)=2.72$, $p=0.144$. Differences between NT and p44Tg were significant at 6–7 months, $F(1,4)=27.38$, $p=0.0064$ and at 9–12 months, $F(1,4)=211.75$, $p=0.0001$. At 2–4 months the differences between NT and p44Tg mice were not significant, $F(1,4)=1.53\times 10^{-5}$, $p=0.9971$.

Figure 2. Characterization of slowly dividing SVZ cells and migrating neuroblasts in p44Tg mice

A. Quantitative analysis of BrdU incorporation in the SVZ 12 days after labeling. Cell proliferation is expressed as the number of BrdU positive cells/mm². Results represent mean $\pm$ S.D. ANOVA revealed a significant main effect of genotype, $F(1,8)=10.73$, $p=0.0113$ and age, $F(1,8)=14.18$, $p=0.0055$. The genotype $\times$ age interaction was not significant, $F(1,8)=4.84$, $p=0.0588$. Simple main effect test showed that there was a significant decrease in the number of proliferating cells with age in the p44Tg mice, $F(1,4)=17.54$, $p=0.0138$. The number of BrdU positive cells was not significantly different between the two age groups in NT mice, $F(1,4)=1.24$, $p=0.3275$. Differences between NT and p44Tg were significant at 9–12 months, $F(1,4)=19.64$, $p=0.0114$. At 2–4 months the differences between NT and p44Tg mice were not significant, $F(1,4)=0.467$, $p=0.5321$. **B.** BrdU, GFAP and DRAQ5 staining of long-term labeled SVZ cells. Upper panel. Low magnification confocal microscope image of a representative coronal section at the level of the lateral ventricle (level C in panel A) from a young NT mouse showing a BrdU positive cell corresponding to a slow dividing SVZ cell (arrow). Scale bar: 20 μ m. Lower panel. Higher magnification analysis of the same BrdU positive cell including individual channels for BrdU (green), GFAP (red) and DRAQ5 (blue), merged images and orthogonal three-dimensional analysis showing a close association between BrdU and GFAP staining. Scale bar: 5 μ m. **C.** Confocal micrograph of a single optical slice shows a BrdU positive slow dividing SVZ cell that is Dlx2 negative. Scale bar: 5 μ m. **D.** Visualization of immature neurons and glial cells in the rostral migratory stream of NT and p44Tg mice. Coronal sections through the rostral migratory stream (RMS) (level B in Fig. 1 A) from 10 month-old mice were triple stained for polysialylated form of neural cell adhesion molecule (PSA-NCAM) (a marker of migrating neuroblasts), glial fibrillary acidic protein (GFAP) (a marker of SVZ and RMS astrocytes) and the nuclear marker DAPI. Immunostaining for PSA-NCAM showed that the population of migrating neuroblasts is dramatically reduced in p44Tg mice. GFAP immunostaining revealed that, although present, astrocyte elements in

the central portion of the RMS are more dispersed in p44Tg brain than in NT brain. Scale bar: 50 μ m.

Figure 3. Quantification of new neurons in the olfactory bulb of NT and p44Tg mice

A. Morphology of the olfactory bulb of NT and p44Tg mice. Shown are representative Nissl-stained coronal sections through the main olfactory bulb (level A in Fig. 1 A) from 9–10 month-old mice. Scale bar: 500 μ m. **B.** Schematic drawing showing the different layers of the olfactory bulb (ONL: olfactory nerve layer; GL: glomerular layer; EPL: external plexiform layer; M/T: mitral/tufted cell layer; IPL: internal plexiform layer GCL: granule cell layer; D: dorsal; V: ventral; M: medial; L: lateral). **C.** Quantification of proliferating cells in the granule cell layer of 9–10 month-old mice. Analysis was performed in the regions indicated by red asterisks in the drawing shown in B. The density of BrdU positive cells in the granule cell layer is significantly reduced in p44Tg mice compared to NT controls. Comparisons were performed using two-tailed Student's *t* test (dorsal: $t(4)=6.224$, $p=0.0034$; medial: $t(4)=6.120$, $p=0.0036$; ventral: $t(4)=5.521$, $p=0.0053$). This result is consistent with the reduced cell proliferation observed in the SVZ of p44Tg mice. **D.** Newly generated neurons in the granule cell layer. Shown are representative confocal microscope images of the dorsal granule cell layer of coronal sections stained for BrdU and the neuronal marker NeuN. Results indicate 96% of BrdU positive cells were also positive for NeuN, suggesting that most newly generated cells differentiated into olfactory bulb neurons in both NT and p44Tg, but the number of new neurons was lower in the latter. Scale bar: 10 μ m.

Figure 4. Neurosphere culture of SVZ cells from NT and p44Tg mice

A. Phase contrast images showing primary neurosphere cultures derived from the SVZ of 6-month-old NT and p44Tg mice. p44Tg NS were smaller than NT controls. Scale bar: 30 μm for 1 and 2 DIV; 100 μm for 3, 5 and 7 DIV. **B.** Quantification of NS diameter in NT, P+/+ and Q+/+ 7-DIV-cultures generated from 6-month-old mice (upper panel), and NT and p44Tg cultures from 2.5-month-old and 11.5-month-old mice (lower panel). Results represent the mean ± S.D. from 3–4 mice. At least 120 NS of each genotype and age were measured, except for the 11.5-month-old group in which 77 NT and 20 p44Tg NS were analyzed. Quantification of body size, expressed as total body weight, of 6-month-old NT (n=11), P+/+ (n=19) and Q+/+ (n=5) female mice (middle panel). Upper panel. We found a significant main effect of genotype, $F(2,7)=7.949$, $p=0.0158$. Tukey/Kramer test showed that the diameter of NS over-expressing p44 (P+/+ and Q+/+ lines) was significantly reduced by 35% relative to NT NS ($p<0.05$). The difference in diameter between P+/+ and Q+/+ NS was not significant ($p>0.05$). Middle panel. We found a significant main effect of genotype, $F(2,32)=12.535$, $p=0.0001$. Tukey/Kramer test showed that the body weight of P+/+ females was significantly smaller than that of NT females ($p<0.05$). On the other hand, body weight of Q+/+ animals was not significantly different compared to NT animals ($p>0.05$). Lower panel. A significant main effect of genotype, $F(1,8)=32.28$, $p=0.0005$, was observed. The effect of age was not

significant, $F(1,8)=0.236$, $p=0.6398$. The age $\times$ genotype was also not significant, $F(1,8)=0.204$, $p=0.6398$. Neurosphere diameter was significantly smaller in P+/+ cells relative to NT cells, both at 2.5 months and 11.5 months ($p<0.05$). **C.** Number of primary NS generated from NT and P+/+ mice at 2.5 and 11.5 months of age. Results represent mean $\pm$ S.D of the number of primary NS generated from 3 mice per group. ANOVA revealed a significant main effect of genotype, $F(1,10)=9.68$, $p=0.0110$ and age, $F(1,10)=10.17$, $p=0.0097$. The genotype $\times$ age interaction was not significant, $F(1,10)=0.019$, $p=0.8927$. Simple main effect test showed that there was a significant decrease with age in the number of NS generated from P+/+ mice $F(1,5)=11.38$, $p=0.0198$. On the contrary, the number of NS generated from NT mice was not significantly different between the two age groups, $F(1,5)=3.47$, $p=0.1211$. We also found a significant reduction in the number of NS generated from 11.5-month-old P+/+ relative to NT controls, even after the data was adjusted for differences in body size, $F(1,4)=445.77$, ($p<0.0001$). At 2.5 months the differences in the number of NS generated from NT and P+/+ mice were not significant, $F(1,6)=3.71$, $p=0.1024$. **D.** Self-renewal, measured as the number of secondary NS/number of plated cells $\times$ 100, showed a significant main effect of genotype, $F(1,8)=26.16$, $p=0.0009$, but not of age, $F(1,8)=4.51$, $p=0.0663$. The genotype $\times$ age interaction was not significant, $F(1,8)=0.166$, $p=0.6942$. Self-renewal was significantly lower in p44Tg cells relative to NT cells, both at 2.5 months and 11.5 months ($p<0.05$). **E.** Amplification, measured as the number of cells obtained from secondary NS/number of plated cells, showed a significant main effect of genotype, $F(1,8)=97.97$, $p<0.0001$. and age, $F(1,8)=7.58$, $p=0.0249$. The genotype $\times$ age interaction was also significant, $F(1,8)=6.159$, $p=0.038$. Amplification =was significantly lower in p44Tg cells relative to NT cells, both at 2.5 months and 11.5 months ($p<0.05$). Although the overall ANOVA showed an age effect, subsequent analysis of each genotype independently failed to show a significant effect of age for either genotype ($p>0.05$). Results in **D** and **E** represent the mean $\pm$ S.D. from 3 mice.

Neurospheres

Olfactory bulb

Figure 5. Detection of apoptotic cells in neurosphere cultures and olfactory bulb sections of NT and p44Tg mice

A. Active caspase-3 staining of intact NT and p44Tg NS. **B.** FISEL staining of NS-dissociated NT and p44Tg cells. No apparent differences in the number of apoptotic cells were revealed by both markers of apoptosis. Scale bar in A and B: 20 μ m. **C.** Quantification of the percentage of active caspase-3 and FISEL positive cells from NT and p44Tg dissociated NS. Results represent mean $\pm$ S.D. The percentage of apoptotic p44Tg cells was not significantly different relative to NT controls (for active caspase-3, $t(4)=2.479$, $p=0.2887$; for FISEL, $t(4)=1.771$, $p=0.1513$). Comparisons were performed using two-tailed Student's t test. **D.** FISEL staining performed on coronal sections through the main OB (level A in Fig. 1 A) from 9–10 month-old mice. Arrows indicate DAPI stained cells that were positive for FISEL. Scale bar: 20 μ m. **E.** Quantitative analysis of apoptosis in the OB. Results are expressed as mean $\pm$ S.D. of the number of FISEL positive cells/mm². The number of FISEL positive cells was not significantly different between the two genotypes (dorsal, $t(4)=0.037$, $p=0.9724$; medial, $t(4)=-0.264$,

p=0.8052; ventral, $t(4) = -0.269$, p=0.8011). Comparisons were performed using two-tailed Student's t test. These results demonstrate that p44 expression does not alter spontaneous apoptosis in SVZ and olfactory bulb cells and support the conclusion that apoptosis does not account for reduced neurogenesis in p44Tg mice.

Figure 6. p21 and phospho-p53 expression in neurospheres derived from NT and p44Tg mice
p21 (A) and phospho-p53 (Ser15) (B) immunofluorescence staining of NT and p44Tg neurospheres. NS were isolated from 6-month-old mice and grown for 7 d. For each NS, a Z-series stack of images was collected at 1- μ m intervals using a standard Zeiss LSM 510 confocal microscope. Top panels: fluorescence images. Bottom panels: overlay of differential interference contrast (DIC) and fluorescence images. Bar: 20 μ m. Graphs show quantification of the fluorescence signal. Intensity level measurements were performed on the images at the center of the stack using the frequency histogram function of the LSM 510 software package (Version 2.8). The fluorescence rate was defined as fluorescence intensity per μ m² and expressed as percentage of NT values. Shown are means $\pm$ S.D. of 5–6 NS for each genotype. p44Tg cells express significantly higher levels of p21 ($t(10)=-5.893$, $p=0.0002$) and phospho-p53 ($t(9)=-3.291$, $p=0.0094$) than NT cells. Comparisons were performed using two-tailed Student's t test.

Figure 7. Cell cycle analysis of NSCs derived from NT and p44Tg mice

A. Cumulative BrdU labeling of NT and p44Tg NSCs at 4 DIV. Shown are means $\pm$ SEM (n=5–9). At least 2,400 cells were counted per genotype per time point. Regression analysis estimated cell cycle times of 20.6 h (NT) and 28.3 h (p44Tg) and S phase duration of 3.0 h (NT) and 5.8 h (p44Tg); $R^2 = 0.9917$ (NT) and $R^2 = 0.9856$ (p44Tg). **B.** Cell cycle profiles of NT and p44Tg NSCs at 7 DIV stained with acridine orange. Data were analyzed with FlowJo software. Shown are results from a representative experiment. **C.** Quantification of cell cycle profiles from independent experiments (NT, n=3; P+/+, n=4). Shown are means $\pm$ SD of the percentage of cells in each phase of the cell cycle as determined by FlowJo software. Comparisons performed using two-tailed Student's *t* test, showed no significant differences

between genotypes for G0/G1, $t(5)=2.5706$, $p=0.3344$; S, $t(5)=2.5706$, $p=0.4324$; G2/M, $t(5)=2.5706$, $p=0.1088$. The percentage of cells in G0 was significantly higher in p44Tg cultures than in NT, $t(5)=2.5706$, $p=0.049$.

Figure 8. Olfactory discrimination assessment of NT and p44Tg mice

Mice (15–16 months old) were tested for their ability to discriminate between two odors, almond and coconut, in order to avoid consuming bitter-tasting water. Results represent the average number ($\pm$ S.E.) of correct responses each genotype made on two sets of tests. Both genotypes performed well on the 100:0 tests, responding correctly on nearly all trials. However, when the task was made more challenging by mixing the odors in a 60:40 ratio to create one stimulus that was mostly almond with some coconut and another that was mostly coconut with some almond, both genotypes were significantly worse at avoiding the bitter water. This decrement of performance was more pronounced in the p44Tg group than in the NT group. Data were analyzed with a repeated measures analysis of variance with genotype (p44Tg vs. NT) as a between group variable and stimulus ratio (100:00 and 60:40) as a within subject variable. The analysis revealed a significant main effect of genotype, $F(1,7)=8.39$, $p=0.02$. Overall, group NT made more correct responses than group p44Tg. The main effect of Extract Ratio was also significant, $F(1,7)=17.42$, $p<0.01$, reflecting greater accuracy on the 100:0 tests than on the 60:40 tests. The genotype $\times$ stimulus ratio interaction was not significant, $F(1,7)=2.92$, $p=0.13$.

Table 1
Cell density in the olfactory bulb of NT and p44Tg mice

	Granule cell layer	Glomerular layer	Mitral/Tufted cell layer
NT	367.7 ± 40.3	346.1 ± 60.3	93.7 ± 22.3
P+/+	267.3 ± 37.3	261.1 ± 22.2	87.8 ± 11.8
<i>P</i> value	0.034	0.0054	0.706
<i>t</i> (df)	<i>t</i> (4)= 3.167	<i>t</i> (4)= 5.489	<i>t</i> (4)= 0.405

Data were obtained from 9–12-month-old NT and p44Tg mice (n=3 mice of each genotype) and represent mean ± standard deviation. Comparisons were performed using two-tailed Student's *t* test.