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INTRACEREBROVENTRICULAR INFUSION OF INSULIN-LIKE GROWTH FACTOR-I AMELIORATES THE AGE-RELATED DECLINE IN HIPPOCAMPAL NEUROGENESIS

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Abstract—The dentate gyrus of the hippocampus is one of few regions in the adult mammalian brain characterized by ongoing neurogenesis. Significantly, recent studies indicate that the rate of neurogenesis in the hippocampus declines with age, perhaps contributing to age-related cognitive changes. Although a variety of factors may influence the addition of new neurons in the adult dentate gyrus, the mechanisms responsible for the age-related reduction remain to be established. Insulin-like growth factor-I (IGF-I) is one promising candidate to regulate neurogenesis in the adult and aging brain since it influences neuronal production during development and since, like the rate of neurogenesis, it decreases with age. In the current study, we used bromodeoxyuridine labeling and multilabel immunofluorescence to assess age-related changes in neuronal production in the dentate gyrus of adult Brown Norway×Fischer 344 rats. In addition, we investigated the relationship between changes in neurogenesis and the age-dependent reduction in IGF-I by evaluating the effect of i.c.v. infusion of IGF-I on neurogenesis in the senescent dentate gyrus. The analyses revealed an age-dependent reduction in the number of newly generated cells in the adult dentate subgranular proliferative zone and, in addition, a 60% reduction in the differentiation of newborn cells into neurons. Restoration of IGF-I levels in senescent rats significantly restored neurogenesis through an approximately three-fold increase in neuronal production.

The results of this study suggest that IGF-I may be an important regulator of neurogenesis in the adult and aging hippocampus and that an age-related decline in IGF-I-dependent neurogenesis could contribute to age-related cognitive changes.

Key words: bromodeoxyuridine, dentate gyrus, aging, rodent, neuronal proliferation, differentiation.

The production of granule neurons in the hippocampal dentate gyrus continues into adulthood in several species, including humans (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999). Precursors divide in the subgranular proliferative zone (PZ) at the border of the granule cell layer (GCL) and the hilus. Within a few weeks, some newborn cells differentiate into neurons, migrate into the GCL, and project to the CA3 region of the hippocampus (Cameron et al., 1993). Several factors appear to influence neuronal turnover in the adult dentate gyrus. For example, neurogenesis declines with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Cameron and McKay, 1999) but is enhanced by environmental enrichment (Kempermann et al., 1997, 1998; Nilsson et al., 1999). Additionally, several studies (Gould et al., 1992; Kuhn et al., 1997; Cameron and Gould, 1994; Cameron et al., 1998a,b; Gould and Tanapat, 1999; Tanapat et al., 1999; Wagner et al., 1999) suggest that multiple growth factors and hormones, including basic fibroblast growth factor (bFGF), epidermal growth factor, adrenal steroids, and estrogen, may influence neuronal production in developing and young adult rodents; possible regulation by such growth factors in aging animals remains unexplored.

Among the growth factors that may regulate neurogenesis, insulin-like growth factor-I (IGF-I) is of particular interest given its pattern of regulation across the lifespan. IGF-I expression closely corresponds to the

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Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; BN×F344, Brown Norway×Fischer 344; BrdU, 5-bromo-2′-deoxyuridine; DAB, 3,3′-diaminobenzidine; DIC, differential interference contrast; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; IGF-I, insulin-like growth factor-I; MANOVA, multiple analysis of variance; NDS, normal donkey serum; NeuN, *neu*ronal *n*uclei; NHS, normal horse serum; PZ, proliferative zone; SSC, saline sodium citrate; TBS, Tris-buffered saline; TBS-Tds, Tris-buffered saline plus 3.0% normal donkey serum/0.2% Triton X-100; TBS-Ths, Tris-buffered saline plus 3.0% normal horse serum/0.2% Triton X-100.

developmental period of widespread neurogenesis (Rotwein et al., 1988; Bondy, 1991; Baker et al., 1993; Anlar et al., 1999). Subsequently, IGF-I gradually declines to a lower, adult level of expression (Rotwein et al., 1988) but is maintained at somewhat higher levels in persistently neurogenic regions such as the hippocampus (Anlar et al., 1999). IGF-I levels undergo a later, second decline (e.g. Sonntag et al., 1999), approximately in parallel with the reported decrease in hippocampal neurogenesis (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998).

Studies of transgenic mice and a small number of experimental studies provide more direct evidence that IGF-I influences the dentate gyrus in developing and young adult rodents. The volume of the dentate GCL and the total number of granule neurons are increased by IGF-I overexpression (O'Kusky et al., 2000) and are reduced by IGF-I gene deletion (Beck et al., 1995; Cheng et al., 2001). Experimentally, peripheral infusion of IGF-I into rats made IGF-I-deficient by hypophysectomy restores production of granule neurons (Åberg et al., 2000). Moreover, IGF-I appears to be a critical mediator of the increase in hippocampal neurogenesis that occurs in response to increased exercise (van Praag et al., 1999; Carro et al., 2000; Trejo et al., 2001). Where IGF-I acts to elicit these effects remains unresolved, as do the critical questions of whether and how IGF-I modulates neurogenesis in the normal adult and aging hippocampus.

This study was designed to test the hypothesis that IGF-I acts directly within the brain to modulate neurogenesis in the dentate gyrus of adult and aging rats, such that the age-related decline in IGF-I leads to a significant decrease in neurogenesis. We used bromodeoxyuridine (BrdU) immunohistochemistry and stereological methods to quantify cell proliferation and survival in the dentate gyrus of young, middle-aged, and old Brown Norway×Fischer 344 (BN×F344) rats. We then identified the phenotype of newborn cells using immunofluorescent labeling for BrdU and cell type-specific markers. In addition to quantifying age-dependent changes, we assessed the effect of i.c.v. infused IGF-I on cell proliferation, survival, and differentiation in the dentate gyrus of old rats in order to evaluate the effects of IGF-I acting directly within the brain.

EXPERIMENTAL PROCEDURES

Animals and surgery

Young (5 months), middle-aged (18 months), and old (28 months) male BN×F344 rats were acquired from the National Institute on Aging Colony at Harlan Industries (Indianapolis, IN, USA). For 4 weeks prior to surgery, animals were housed individually under a 12-h light–dark schedule in a climate-controlled room. Food and water were provided *ad libitum*. The animal facility at the Wake Forest University School of Medicine is fully accredited by the American Association for Accreditation of Laboratory Animal Care and complies with all Public Health Service–National Institutes of Health and institutional policies and standards for laboratory animal care. All protocols described herein were approved by the institutional Animal Care and Use Committee.

For i.c.v. infusion of IGF-I or vehicle, each rat was anesthetized using ketamine (90 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). A 28-gauge stainless steel cannula was implanted into the right lateral ventricle (Alzet® brain infusion kit; Alza Corporation, Mountain View, CA, USA; bregma −0.8 mm, lateral 1.4 mm, Paxinos and Watson, 1986) and connected to an Alzet® osmotic minipump (Alza Corporation), which was placed subcutaneously in the neck/shoulder region. One half of the old rats received recombinant human IGF-I (Bachem California, Torrance, CA, USA) delivered at a rate of 50 ng/0.5 µl/h. The remaining old rats and all middle-aged and young animals served as controls and were infused with vehicle (saline). Pumps were replaced at 14-day intervals.

Starting on the seventh day of infusion, rats were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg body weight, in saline plus 0.007 N NaOH) twice daily (9.00, 17.00 h) for five consecutive days. We used multiple injections of BrdU over several days in order to assure adequate numbers of labeled cells for accurate quantitation in old animals and to reduce any possible effects of age- and/or treatment-dependent differences in the length of any phase of the cell cycle (discussed in Åberg et al., 2000). One half of the animals (short-term survival group) were killed on day 14, 3 days after the last BrdU injection. With this survival period, differences among animals in the number of BrdU-labeled cells reflect primarily differences in the rate of proliferation (Kempermann et al., 1997, 1998; Åberg et al., 2000). In order to assess effects of age and IGF-I on the survival and differentiation of newly generated cells, the remaining animals (long-term survival group) continued infusion of IGF-I or vehicle for an additional 28 days and were killed on day 42, a total of 31 days after the last BrdU injection. The experimental design is depicted in Fig. 1.

At sacrifice, each animal was deeply anesthetized with pentobarbital and perfused intracardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed overnight in 4% paraformaldehyde, cryoprotected through graded sucrose, blocked at the optic chiasm, and embedded for frozen sectioning. Coronal cryostat sections (40 µm) through the extent of the dentate gyrus were cut and stored at −20°C in cryoprotectant solution (25% ethylene glycol, 25% glycerol, 50% 0.1 M phosphate buffer, pH 7.4) until processed for immunohistochemistry or immunofluorescence.

Antibodies and immunochemicals

Monoclonal mouse anti-BrdU (1:150; Becton Dickinson, San Jose, CA, USA) and biotinylated horse anti-mouse IgG (1:300; Vector Laboratories, Burlingame, CA, USA) were used for immunohistochemical identification of BrdU-labeled cells. For triple immunofluorescent labeling, monoclonal rat anti-BrdU (1:200; Harlen-Sera Lab, Loughborough, UK) was substituted for mouse anti-BrdU and combined with monoclonal mouse anti-neuronal nuclei (NeuN; 1:2000; Chemicon, Temecula, CA, USA), a marker of neurons (Mullen et al., 1992), and polyclonal rabbit anti-glial fibrillary acidic protein (GFAP; 1:150; Chemicon), a marker of astrocytes. The following fluorophore-conjugated secondary antibodies were used for detection of BrdU-labeled, NeuN-positive, and GFAP-labeled cells, respectively: donkey anti-rat IgG-Cy3 (1:300; Jackson ImmunoResearch, West Grove, PA, USA), goat anti-mouse IgG-Alexa 488 (1:300; Molecular Probes, Eugene, OR, USA), and donkey anti-rabbit IgG-Cy5 (1:300; Jackson ImmunoResearch).

Immunohistochemistry

Free-floating sections were transferred from cryoprotectant to a 24-well plate containing 2×SSC (0.3 M NaCl and 0.03 M sodium citrate). For DNA denaturation, sections were incubated for 2 h in 50% 2×SSC/50% formamide at 65°C, rinsed twice in 2×SSC, incubated in 2 N HCl for 30 min at 37°C, and then rinsed twice in 0.1 M borate buffer (pH 8.5). After replacing the borate buffer with Tris-buffered saline (TBS, 0.15 M NaCl and 0.1 M Tris–HCl, pH 7.5), endogenous peroxidase

activity was blocked by incubating sections in 0.6% H_2O_2 in TBS for 30 min. Following two washes in TBS, sections were incubated for 1 h in 3%

bined PZ/GCL were scored for colabeling with NeuN, GFAP, or neither label. We combined the PZ and GCL regions for this analysis given the possibility that newborn neurons may differentiate and begin expressing neuron-specific markers prior to migrating out of the PZ (as was observed in many animals). For the young animals, in which the density of BrdU labeling was high, the right hemisphere in every 25th section from each animal was analyzed for colocalization of BrdU and NeuN or GFAP. For the middle-aged and old animals, in which the density of BrdU labeling was lower, every 12th section per animal was analyzed. A total of 28–161 BrdU-labeled cells per animal were examined for coexpression of the cell type-specific markers. This analysis yielded the percentage of BrdU-positive cells colabeled with (1) NeuN, (2) GFAP, or (3) neither NeuN nor GFAP. For each animal surviving 31 days after the last BrdU injection, the percentage value for BrdU-positive cells colabeled with NeuN was used to derive the total number of newborn neurons in the PZ/GCL.

Statistical analysis

Of the 50 animals initially in the study, five control rats died during the course of the study (one young, two middle-aged, and two old). One old control rat and one IGF-I-treated rat were eliminated because of evidence of disease at necropsy. In addition, one or two rats were excluded from six of the eight groups because lost or damaged sections precluded complete sampling through the dentate gyrus that was comparable to that in the other animals. For the final analyses the short-term survival group included five young, four middle-aged, and three old control rats, as well as four old, IGF-I-treated animals. The long-term survival group included five young, five middle-aged and four old control rats, as well as six old, IGF-I-treated animals. Statistical analyses were performed using SYSTAT 7.0.1 statistical system software (SPSS, Chicago, IL, USA). Comparisons of BrdU-labeled cell numbers and regional volumes measured in the GCL, PZ, and hilus were made with respect to animal age or IGF-I treatment. Effects of age on these six parameters were determined for animals in each group (short- or long-term survival) using one-factor multiple analysis of variance (MANOVA) with one independent variable (age) and six dependent variables (number of BrdU-labeled cells in each of the GCL, PZ, and hilus and volume of each of the three regions). A significant result, as indicated by a Wilks' Lamba F statistic with a probability < 0.05, was followed by univariate analysis of variance (ANOVA) for each dependent variable and, when appropriate, Tukey comparisons. Effects of vehicle versus IGF-I infusion in old rats were determined for the same set of parameters using Student's t-tests.

To test for specific effects of age or IGF-I treatment on the survival of newborn cells, the mean numbers of BrdU-labeled cells in animals from the long-term survival group (killed 31 days after the last BrdU injection) were divided by the equivalent counts from rats in the short-term survival group (killed 3 days after the last BrdU injection). This revealed what fraction of BrdU-labeled cells present shortly after labeling remained present several weeks later. For this analysis, counts in the PZ and GCL were combined in order to avoid the confounding issue of possible differences in the rate of cell migration.

The percentage of BrdU-labeled cells in the PZ/GCL that differentiated into neurons (i.e. that colocalized NeuN) was determined for each animal in the long-term survival group using the immunofluorescently labeled sections. Estimates of the total number of newborn neurons in the PZ/GCL of each rat were then derived by multiplying these percentages by the counts of BrdU-labeled cells in the PZ/GCL from the same animals. Effects of age or IGF-I treatment on neuronal addition were determined using MANOVA or t-tests, respectively, as described above.

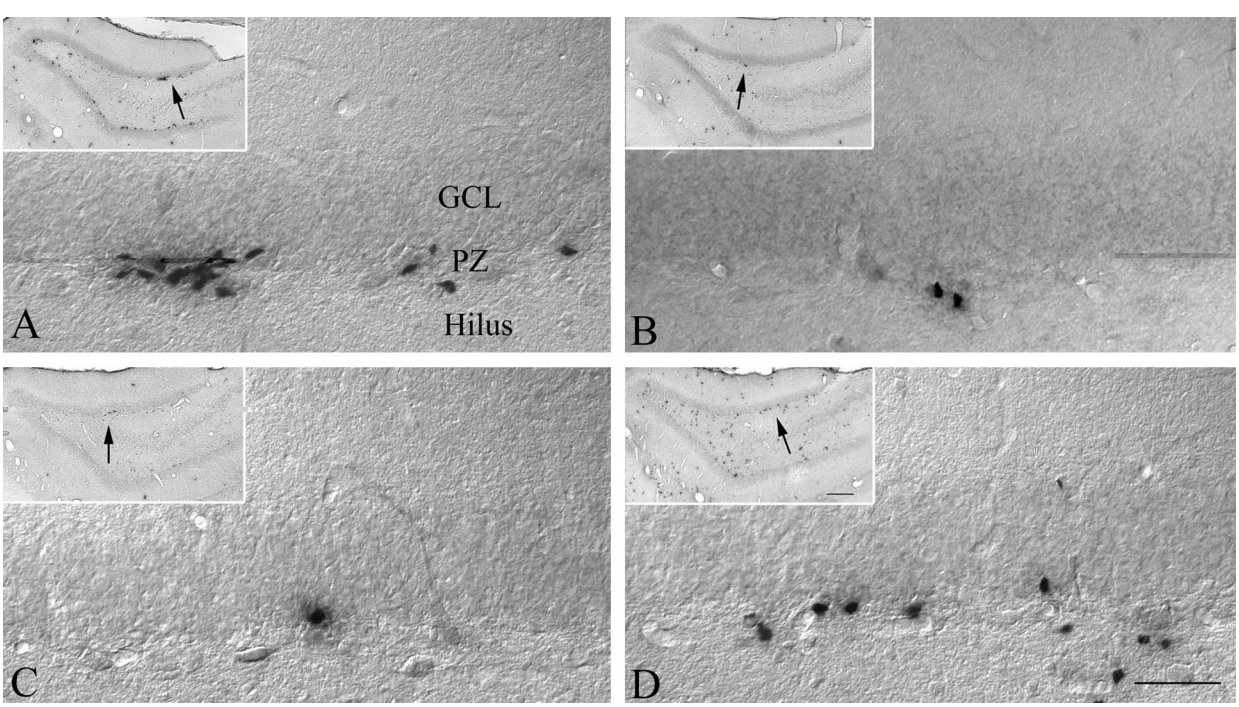

Fig. 2. Immunohistochemical identification of newborn cells in the dentate gyrus. BrdU-labeled cells are evident in representative DIC photomicrographs of the dentate gyrus (insets) in (A) young, (B) middle-aged, (C) old, and (D) old IGF-I-treated animals in the short-term survival group. The arrows within the insets indicate the areas shown in the higher magnification micrographs. Whereas few BrdU-labeled cells are evident in middle-aged and old saline-infused rats (B and C, respectively), many BrdU-positive cells are apparent in young saline-infused and old IGF-I-treated animals, particularly in the PZ (A and D, respectively). Scale bar = 50 μm (inset scale bar = 200 μm). For presentation purposes, adjustments were made to brightness and contrast.

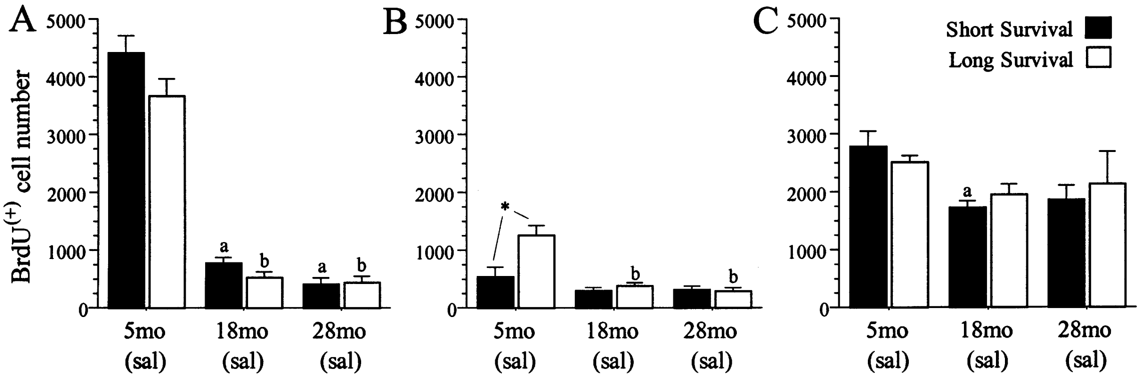

Fig. 3. The number of BrdU-labeled cells decreases with age. (A) An age-related reduction in the number of BrdU-labeled cells in the PZ was apparent in both the short-term (black bars) and the long-term (white bars) survival groups of saline-infused rats. Values presented are mean ± S.E.M. $^{a,b}P < 0.001$ versus 5-month-old rats, post-hoc Tukey test. (B) The number of BrdU-labeled cells did not change with age in the granule cell layer of rats killed 3 days after the last BrdU injection (black bars). Thirty-one days after the final BrdU injection (white bars), however, an age-related decline in the number of newly generated cells was apparent, presumably due to migration of newborn cells from the PZ into the GCL during the longer survival interval. Values presented are mean ± S.E.M. $^{b}P < 0.001$ versus 5-month-old rats, post-hoc Tukey test. $*P < 0.05$, short-term to long-term survival, *t*-test0.0C,9785-54(bn0978 -54(m1t20T9ITD](n54)e483(tul38u)679(best)-47u38u)-4954(edU-labeled)-506(arlls)-5324re

RESULTS

In this study, we quantified the effects of age and IGF-I on: (1) the regional volume of the PZ, GCL, and hilus; (2) the number of newly generated cells present 3 days after BrdU injection (to assess effects on proliferation, at least primarily); (3) the number of newborn cells present 31 days after BrdU injection (to determine effects on survival); and (4) the percentage of newborn cells that differentiated into neurons.

The effects of age and IGF-I on regional volume within the dentate gyrus

Regional volumes were compared among saline-infused rats at 5, 18, and 28 months of age. Age had no effect on the mean volume of the PZ, the GCL, or the hilus. Comparing regional volumes in old rats after 6 weeks of i.c.v. infusion of IGF-I or saline revealed that IGF-I treatment did not alter the volume of any region of the dentate gyrus (data not shown).

The influence of age and IGF-I on the generation of new cells in the dentate gyrus: short-term effects

BrdU-positive cells were present in every animal in each of the three dentate regions examined (Fig. 2). BrdU-labeled nuclei in the PZ generally were small in size (∼10 μm in diameter) and round or oval in shape, consistent with the morphological characteristics of granule cell precursors (Gould et al., 1997). The highest density of BrdU labeling was seen in the PZ, and the majority of labeled cells in the GCL were located close to the PZ. BrdU-positive cells were evenly distributed throughout the hilus. Throughout the dentate gyrus there appeared to be substantially fewer BrdU-labeled cells in older (18- and 28-month-old) animals than in 5-month-old rats.

Quantitative analyses verified that the number of BrdU-labeled cells in animals killed 3 days after the last BrdU injection (short-term survival group) decreased significantly with age (Fig. 3, black bars). The number of BrdU-labeled cells in the PZ (Fig. 3A) was approximately 80% lower in 18-month-old rats than in 5-month-old animals; a trend toward a further decline by 28 months of age did not reach statistical significance. In the hilus (Fig. 3C), the number of BrdU-labeled cells remained relatively stable with age, although there was a small but significant decrease between 5 and 18 months of age. At all ages examined, the number of BrdU-labeled cells in the GCL (Fig. 3B) was modest at 3 days after BrdU injection; no age-related decline was apparent.

To test for short-term effects of IGF-I on newborn cells, the number of BrdU-labeled cells in the dentate gyrus of old rats infused with IGF-I and killed 3 days after the last BrdU injection was compared to the number of BrdU-labeled cells in saline-infused, age-matched controls. IGF-I approximately tripled the number of BrdU-labeled cells in the PZ and in the hilus (Fig. 4A,C, black and black hatched bars). There appeared to be a trend toward an IGF-I-induced increase in the number of BrdU-labeled cells in the GCL after the short-term survival period (Fig. 4B), possibly due to the migration of cells labeled by the first BrdU injections, but the effect did not reach statistical significance.

The influence of age and IGF-I on the generation of new cells in the dentate gyrus: long-term effects

Whereas the short-term survival group allowed assessment of the effects of age and IGF-I on the proliferation (at least primarily) of newly generated cells, the long-term survival group facilitated analysis of the survival of newborn progeny. As in the short-term survival

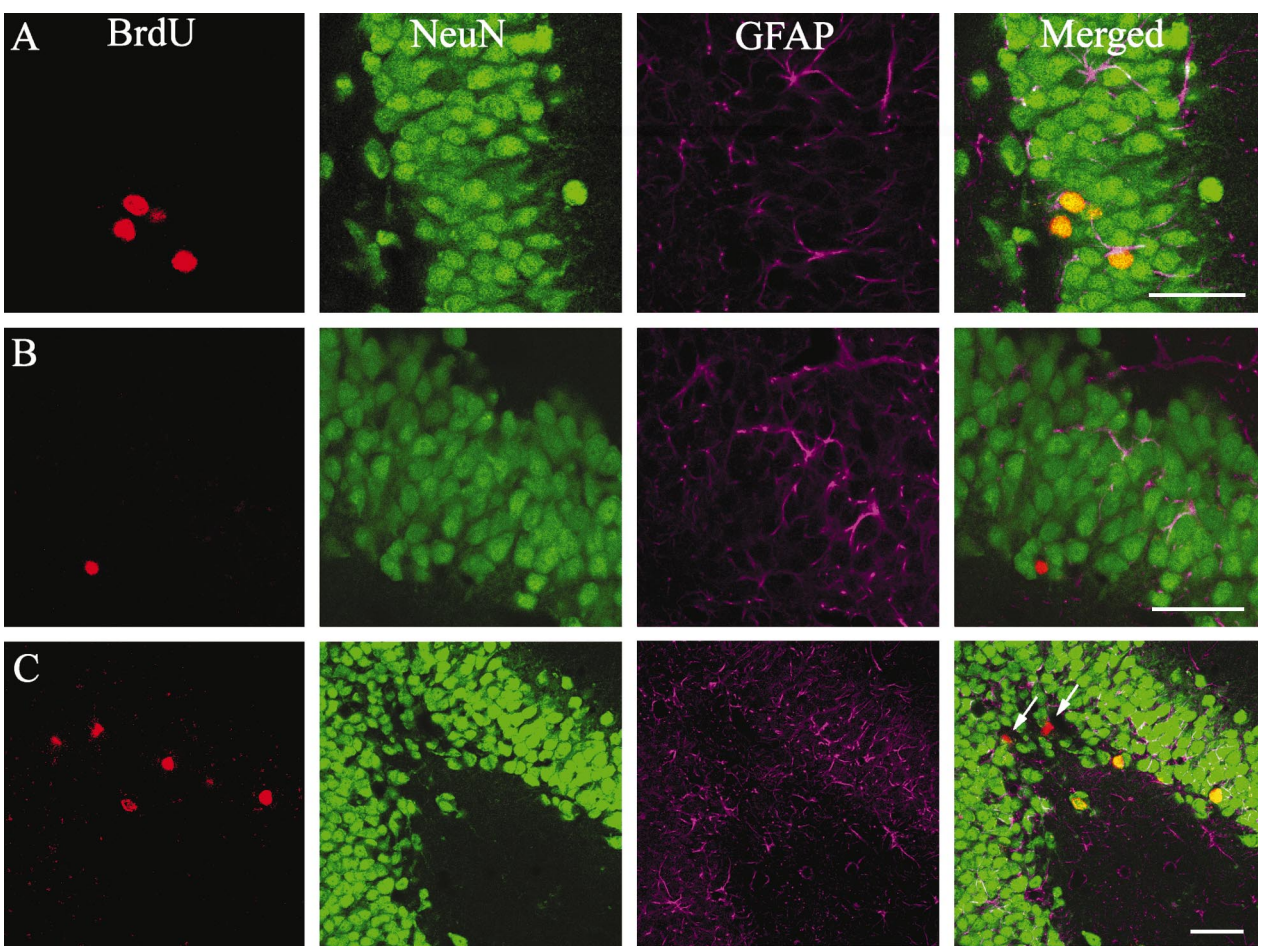

Fig. 5. Immunofluorescent identification of newborn neurons in the dentate gyrus. Triple immunofluorescent labeling for BrdU (red) and the cell-specific markers NeuN (green) and GFAP (purple) was analyzed by laser scanning confocal microscopy. (A) A photomicrograph of a young, saline-infused rat shows colocalization of BrdU and NeuN (yellow cells in merged image), indicating the differentiation of newborn cells into neurons. (B) A photomicrograph of an old, saline-infused rat shows a single newborn cell that is neither a neuron nor an astrocyte. (C) A photomicrograph of an old, IGF-I-treated rat shows five BrdU-labeled cells, three of which are colabeled with NeuN (yellow cells in merged image) and two of which are labeled with neither the neuronal nor the glial marker (arrows). Scale bar = 30 μm. For presentation purposes, adjustments were made to brightness and contrast; individual confocal images are not meant to represent the relative number of newborn cells.

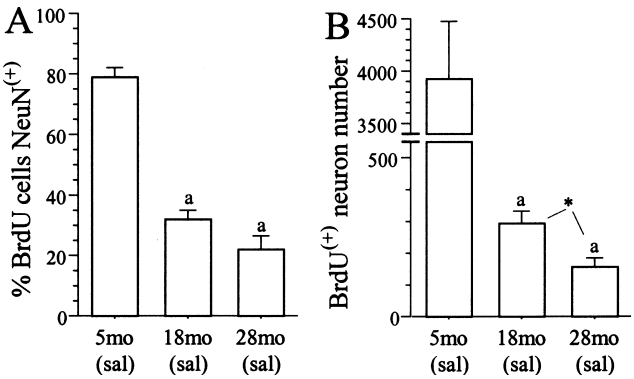

Fig. 6. Neuronal differentiation declines with age. (A) The percentage of BrdU-labeled cells in the combined PZ/GCL that colocalized NeuN decreased significantly between 5 and 18 months of age. (B) Expressing the data as the estimated total number of newly generated neurons (colabeled with BrdU and NeuN) revealed an additional decline in neuronal differentiation between 18 and 28 months of age. Values presented are mean ± S.E.M. $^{a}P < 0.001$ versus 5-month-old animals, ANOVA; $^{*}P < 0.05$, 18 versus 28 months of age, *t*-test.

group, age-dependent differences in the number of BrdU-labeled cells were apparent in animals killed 31 days after the last BrdU injection. At that time, the number of BrdU-labeled cells in the PZ was approximately 85% lower in 18-month-old animals than in 5-month-old rats (Fig. 3A, white bars). No statistically significant additional decline was apparent between 18 and 28 months of age. In the hilus, there was no difference between young and older animals in the number of BrdU-labeled cells present 31 days after BrdU injection (Fig. 3C, white bars). With the longer survival period, a clear effect of age on the number of BrdU-labeled cells in the GCL became apparent (Fig. 3B, white bars). The number of newborn cells in the GCL was approximately 70% lower at 18 months of age than in 5-month-old rats. Thus, age-dependent differences in the GCL were revealed after an interval sufficient to allow newly generated cells to migrate into the GCL.

Differences among age groups in the number of BrdU-labeled cells after the longer survival period might have arisen simply as a result of the differences in cell proliferation or also might reflect age-dependent differences in cell survival. To better assess the latter, we expressed the mean number of BrdU-labeled cells at each age in the long-term survival group (Fig. 3, white bars) as a percentage of that in the short-term survival group (Fig. 3, black bars). Since all newborn cells may not migrate at the same rate, we determined the number of labeled cells in the combined PZ and GCL regions for this analysis. This analysis revealed no effect of age on the rate of survival in either the PZ/GCL or in the hilus. Moreover, similar percentages of newborn cells survived in the two regions.

I.c.v. infusion of IGF-I maintained an approximately three-fold increase in the number of BrdU-labeled cells in the PZ, GCL, and hilus in old rats examined 31 days after BrdU injection (Fig. 4, white and white hatched bars). Expressing the BrdU-labeled cell counts in IGF-I-treated rats in the long-term survival group (Fig. 4, white hatched bars) as a percentage of that in the short-term survival group (Fig. 4, black hatched bars) revealed that IGF-I did not significantly alter survival of newly generated cells in the PZ/GCL region or in the hilus.

The effects of age and IGF-I on neuronal differentiation in the dentate gyrus

Figure 5 includes representative confocal photomicrographs of triple-labeled sections from young (Fig. 5A) and old (Fig. 5B) control rats and from old IGF-I-treated animals (Fig. 5C). At all ages, less than 1% of all BrdU-labeled cells colocalized with the astroglial marker GFAP; indeed, in many animals no GFAP-positive, BrdU-labeled cells were observed. The fate of cells in the hilus was not quantified but qualitative observations revealed no newly generated neurons within this region. In contrast, newborn neurons could be identified in the PZ and GCL in every animal at each age. In young rats, an average of 79% of BrdU-labeled cells in the PZ and GCL were labeled with NeuN. The percentage of newborn cells that differentiated into neurons was approximately 60% lower in 18-month-old rats than in 5-month-old animals (Fig. 6A). There appeared to be an additional decrease between 18 and 28 months of age in the percentage of BrdU-labeled cells that colocalized NeuN but that change was not, in itself, statistically significant. Additional analysis indicated, however, that

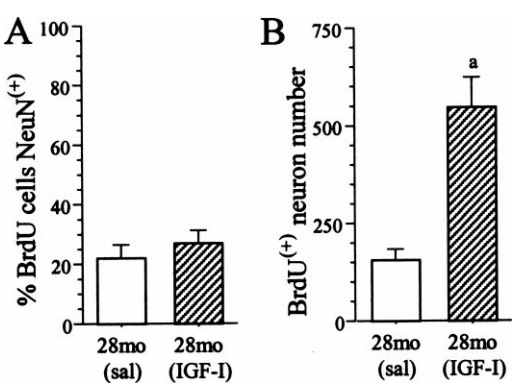

Fig. 7. IGF-I treatment does not influence neuronal differentiation. (A) IGF-I treatment had no effect on the percentage of newborn cells in the combined PZ/GCL that differentiated into neurons. (B) The total number of newly generated neurons was dramatically increased in old IGF-I-treated rats (white hatched bars) versus age-matched controls (white bars). Values presented are mean ± S.E.M. $^{a}P < 0.01$ versus 28-month-old saline-infused rats, *t*-test.

there was a decline in neurogenesis between 18 and 28 months of age. When the total number of newborn neurons was calculated by multiplying the number of BrdU-labeled cells by the percentage that were NeuN-positive, a decline of approximately 50% was observed between middle-aged and old rats (Fig. 6B). ANOVA verified the large decline in neuron number (>10-fold) between young rats and each of the two older age groups ($P<0.001$). In that analysis, the large magnitude of the change from young to middle-aged limited the ability of the test to measure the smaller change between middle-aged and old animals. When middle-aged and old rats were compared directly, an additional decline in neurogenesis from middle age to later senescence was apparent ($P<0.05$, Student's t-test).

Overall, significantly more newborn neurons were found in IGF-I-treated rats than in age-matched controls ($P<0.01$, t-test, Fig. 7B). IGF-I did not, however, selectively induce a neuronal fate since the percentage of BrdU-labeled cells in IGF-I-treated animals that colocalized NeuN was identical to that observed in age-matched controls (Fig. 7A). Thus, the approximately three-fold increase in the total number of newborn neurons in old IGF-I-infused rats compared to age-matched controls (Fig. 7B) was accounted for by effects on cell proliferation (i.e. changes following a short-term survival period) rather than survival or differentiation.

DISCUSSION

Regulation of neurogenesis in the adult dentate gyrus involves control of at least three cellular processes: proliferation, survival, and differentiation. Each of these may be regulated, to some extent, independently and may be subject to different age-dependent changes. In this study, we examined the effects of age and IGF-I on the number of BrdU-labeled cells following a short-term survival period to assess cell proliferation and following a long-term survival period to assay cell survival and differentiation. This study thus addressed each aspect of neurogenesis in order to test whether and how direct i.c.v. infusion of IGF-I influences neurogenesis in the normal adult and aging brain.

Age-dependent changes in neurogenesis

Taken together, our results and those of previous studies indicate that neurogenesis declines with age (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Cameron and McKay, 1999) due to specific decreases in the proliferation of precursors and in the percentage of new cells that differentiate into neurons. Our analysis of BN$\times$F344 rats parallels previous studies of other rodent strains, which demonstrated that cell proliferation in the adult dentate gyrus declines 60–80% with age in F344 rats (Kuhn et al., 1996), in Sprague–Dawley rats (Cameron and McKay, 1999), and in C57BL/6 mice (Kempermann et al., 1998). Our observation that cell survival per se is not influenced by age also is consistent with earlier studies and indicates that the age-related reduction in cell proliferation accounts for much of the age-dependent decline in neurogenesis (Kuhn et al., 1996; Kempermann et al., 1998; Cameron and McKay, 1999). Clearly, however, the decline in proliferation is compounded by changes in the differentiation of new cells, such that a much smaller percentage of newborn cells differentiate into neurons in older animals. The fate of the newborn, non-neuronal cells remains unclear. The present study indicates that few develop into astrocytes; additional studies will be required to determine whether the cells remain undifferentiated or develop into microglia and/or oligodendrocytes.

Although this and previous studies provide consistent and comparable evidence for age-related changes in proliferation and differentiation, the time course over which these changes occur is not yet fully clear. Seki and Arai (1995) reported a gradual age-dependent decrease in newborn granule cells over more than a year in Wistar rats. Kuhn et al. (1996) reported that, in F344 rats, the age-related reduction in the density of BrdU-labeled cells was apparent by 12 months of age, with no additional decline during later adulthood and senescence. The present study provides evidence for an additional, albeit less dramatic, decline with later senescence since the number of newborn neurons was significantly lower in 28- than in 18-month-old rats. Whatever the exact time course of these changes, however, it is clear that the decline in neurogenesis, like the age-related change in IGF-I, is not associated simply with the very end of the lifespan.

The effect of IGF-I on neurogenesis in the senescent dentate gyrus

Our results demonstrate that IGF-I significantly reverses the age-related decline in hippocampal neurogenesis and triples the number of newborn neurons in aged rats through a dramatic increase in cell proliferation. One might expect IGF-I to also promote survival, given evidence of its anti-apoptotic actions in many systems (e.g. D'Mello et al., 1993; Takadera et al., 1999; Mason et al., 2000; Niikura et al., 2001; see also Cheng et al., 2001). Of the three aspects of neurogenesis (proliferation, survival, and differentiation), it is most difficult to assess and quantitate experimental effects on survival. The present study suggests, however, that IGF-I has little, if any, effect on the survival of newborn cells in the aged hippocampus, at least during the first 4–5 weeks after their birth. Given evidence that older dentate granule cells may be more vulnerable to cell death than are young neurons (discussed in Gould and Cameron, 1996), an effect of IGF-I on cell survival might be seen only after a much longer survival period than was employed in this study.

The present results extend earlier studies of the effects of IGF-I on neurogenesis in the hippocampus by examining the effects of IGF-I delivered to the brain of normal animals. Significantly, it appears that the exact effects of IGF-I on hippocampal neurogenesis may depend on where IGF-I is delivered and/or on the age of the animal. Systemic and central delivery appear to

produce somewhat different effects despite the fact that IGF-I crosses the blood–brain barrier (Reinhardt and Bondy, 1994; Armstrong et al., 2000; Pan and Kastin, 2000; Pulford and Ishii, 2001). IGF-I deficiency in young rats, created by removal of the pituitary, results in decreased proliferation in the dentate gyrus and a decline in the percentage of newborn cells that differentiate into neurons. Restoration of systemic IGF-I by peripheral injection reverses the decline in neurogenesis, with effects on both proliferation and neuronal differentiation (Åberg et al., 2000). In contrast, the present study demonstrates that IGF-I, delivered directly to the ventricles of normal senescent rats after endogenous IGF-I levels have declined, promotes proliferation but not neuronal differentiation. It is not clear to what extent an IGF-I deficiency created in a young animal by removal of the pituitary can be compared to the normal, age-related decline in IGF-I. It is possible, however, that differentiation may be influenced through effects of IGF-I outside of the brain, and proliferation through direct and specific activity within the hippocampus. Alternatively, IGF-I may have different effects in early versus later adulthood. There is evidence from developmental and *in vitro* studies that IGF-I influences cell survival and differentiation through multiple transduction pathways (e.g. Feldman et al., 1997; Torres-Aleman et al., 1998; Bonni et al., 1999; Petley et al., 1999; Morrione et al., 2000). IGF-I may influence the various phases of neurogenesis (proliferation, survival, differentiation) through different transduction pathways, which may become differentially affected during aging.

The method of IGF-I infusion used in this study was shown previously to fully restore IGF-I levels within the hippocampus (Sonntag et al., 2000). It did not, however, fully restore neurogenesis. Age-related changes in IGF receptors (e.g. D'Costa et al., 1993) or in IGF-I signal transduction may limit the efficacy of IGF-I in old animals. It is also likely, however, that other growth factors contribute to the modulation of neurogenesis in the adult hippocampus. For example, bFGF has been reported to increase cell proliferation and neuronal differentiation in the hippocampus during development and in early adulthood (Kuhn et al., 1997; Tao et al., 1997; Palmer et al., 1999; Tropepe et al., 1999; Wagner et al., 1999). In addition to promoters of neurogenesis like IGF-I and bFGF, there appears to be negative regulation of hippocampal neurogenesis by adrenal steroids. Neuronal proliferation is inhibited by treatment with exogenous corticosterone, while adrenalectomy dramatically increases neurogenesis (Gould et al., 1992; Gould and Cameron, 1996; Cameron et al., 1998a; Cameron and McKay, 1999). Adrenal hormones appear to mediate deleterious effects of stressful experiences, which suppress neurogenesis in the adult dentate gyrus (Gould et al., 1997; Gould and Tanapat, 1999). Furthermore, adrenal steroid levels are low during the first two postnatal weeks, the period during which the majority of granule cells are generated (Sapolsky and Meaney, 1986; Gould and Cameron, 1996), whereas older animals experience more sustained stress-induced increases in adrenal steroids than do young animals (Sapolsky et al., 1983). Thus, IGF-I and adrenal steroids may work antagonistically within the hippocampus to regulate the level of neurogenesis.

Whatever the effects of other factors, the time course of age-related changes in cell proliferation and neurogenesis in the hippocampus appears to correlate with the temporal pattern of changes in the levels of IGF-I within the plasma and brain. In adult BN×F344 rats, plasma IGF-I levels decline between 8 and 14 months of age, remain stable until approximately 22 months of age, and then decline further by 30 months of age (Sonntag and Boyd, 1988). IGF-I levels within the brain are stable from 10 to 24 months of age (levels in younger adults are not known), but then decline 35% by 32 months of age (Sonntag et al., 1999). Thus, the pattern of age-related changes in IGF-I levels appears to include an initial decline in IGF-I in middle age, at least within the plasma, which is followed by a later, secondary fall in plasma and brain IGF-I. The latter decline is also accompanied by a decline in IGF-I receptor number (D'Costa et al., 1993).

The correlation between age-related changes in IGF-I signaling and the decline in neurogenesis reported here and in other studies suggests a significant relationship between IGF-I levels and levels of neurogenesis. Furthermore, the observation that restoring IGF-I in aged animals increases proliferation demonstrates directly that IGF-I is *sufficient* to reverse the age-related decline in neurogenesis. Establishing whether endogenous IGF-I is *necessary* for maintaining neuronal replacement in the adult hippocampus will require additional experiments to evaluate the effects on neurogenesis of blocking IGF-I activity in young adults, before IGF-I levels have declined. Such studies also will address the possibility that IGF-II and the type II IGF receptor are involved in the regulation of neurogenesis, either during normal aging or after IGF-I infusion. IGF-I and -II can activate the heterologous receptors (and the insulin receptor) when present at high concentrations (Rechler and Nissley, 1985; Neely et al., 1991). Like IGF-I, IGF-II declines with age (Park and Buetow, 1991; Kitraki et al., 1993) and can promote neurogenesis, at least in some species (e.g. Zackenfels et al., 1995; Holzenberger et al., 1997; Boucher and Hitchcock, 1998). Thus, either or both IGFs might be involved in modulating hippocampal neurogenesis.

Significance of age and IGF-I-dependent modulation of neurogenesis

The volume of the GCL and the total number of dentate granule cells appear to be essentially stable across the lifespan (see above and West, 1993; Amenta et al., 1995; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Kempermann et al., 1998). Thus, if changes in neurogenesis contribute to age-related cognitive declines, it is likely due to restrictions on neuronal turnover, rather than a reduction in neuron number per se. The present study reveals that, despite dramatic effects on neurogenesis, the total volume of the GCL is unaffected by IGF-I treatment. This suggests that IGF-I acts across

the lifespan to modulate the rate of replacement of dentate granule cells rather than the size of the population. Significantly, recent studies indicate that it may be the ongoing replacement of granule neurons, rather than the absolute number, that is significant for hippocampal function (Lemaire et al., 2000) and that blocking the addition of new neurons in the adult dentate gyrus decreases performance on some hippocampus-dependent tasks (Shors et al., 2001). Thus, IGF-dependent modulation of neurogenesis in the dentate gyrus may contribute significantly to the age-related decline in performance on hippocampus-dependent cognitive tasks, and is likely to play a role in the ability of IGF-I to restore function on such tasks in senescent animals (Markowska et al., 1998; Thornton et al., 2000).

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