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Neurons Generated in Senescence Maintain Capacity for Functional Integration

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Abstract

Adult-born neurons in the dentate gyrus (DG) can survive for long periods, are capable of integrating into neuronal networks, and are important for hippocampus-dependent learning. Neurogenesis is dramatically reduced during senescence, and it remains unknown whether those few neurons that are produced remain capable of network integration. The expression of Arc, a protein coupled to neuronal activity, was used to measure activity among granule cells that were labeled with BrdU four months earlier in young (9 months) and aged (25 months) Fischer344 rats. The results indicate that while *fewer* cells are generated in the senescent DG, those that survive are (a) more likely to respond to spatial processing by expressing Arc relative to the remainder of the granule cell population, and (b) equally responsive to spatial exploration as granule cells of the same age from young animals. These findings provide compelling evidence that newborn granule cells in the aged DG retain the capacity for participation in functional hippocampal networks.

Keywords

Aging; Neurogenesis; Arc; Dentate Gyrus

INTRODUCTION

It is now well established that new neurons can be born and survive in the adult dentate gyrus (DG) of mammals (for review, see Deng et al., 2010). Newly generated granule cells extend axons to CA3 pyramidal cells (Stanfield and Trice, 1988; Hastings and Gould, 1999), and respond in a similar manner to mature granule cells both *in vitro* (Song et al., 2002; van Praag et al., 2002) and *in vivo* (Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007). While the data from some experiments are consistent with the notion that neurogenesis plays a specific role in learning and memory, other studies suggest that it does not. Although a number of explanations for these discrepancies exist, one is that most studies do not directly assess the function of adult-born neurons after they integrate into the neural network (Deng et al., 2010). In fact, determining whether adult-generated cells participate in behaviorally-relevant networks has only recently become possible by assessing

the expression of immediate-early genes such as c-fos (Jessberger and Kempermann, 2003; Kee et al., 2007; Stone et al., 2010), zif268 (Tashiro et al., 2007), and Arc (Ramirez-Amaya et al., 2006; Kee et al., 2007; Alme et al., 2010). Using Arc as a marker that is tightly coupled to neuronal activity (Ramirez-Amaya et al., 2005) and essential to enduring plasticity and memory (Guzowski et al., 2000; Plath et al., 2006), it has been shown that mature newborn granule cells respond to discrete spatial learning behaviors (Ramirez-Amaya et al., 2006; Kee et al., 2007; Stone et al., 2010). In fact, adult-generated cells may remain far more likely to respond to spatial processing than neonatally-generated counterparts, suggesting that they provide a unique contribution to hippocampal-dependent learning (Ramirez-Amaya et al., 2006; Kee et al., 2007; but see Stone et al., 2010). Over the course of progressive aging, which is associated with impaired memory and DG dysfunction (Small et al., 2004; Penner et al., 2010; Marrone et al., 2010a,b), the production of new granule cells is dramatically reduced (e.g., Kuhn et al., 1996; Bizon and Gallagher, 2003; Drapeau et al., 2003; Bizon et al., 2004; Montaron et al., 2006). Whether these deficits may be at least partially mediated by impaired functional integration in addition to a reduction in the number of neurons generated during senescence, however, remains unknown.

Addressing this issue is critical because, although there are discrepant results (e.g., Bizon and Gallagher, 2003; Drapeau et al., 2003), at least one study suggests that, although neurogenesis is dramatically reduced in all aged animals, those aged animals that show the *greatest decrease* in neurogenesis also show *superior* performance in learning and memory tasks (Bizon et al., 2004). This finding raises the possibility that newly generated neurons may lose their ability to integrate properly into the network with progressive age. If the few neurons that are generated during senescence are functionally aberrant, this may inhibit, rather than promote, DG function. Certainly different therapeutic approaches would be suggested if granule cells generated during senescence maintain proper cellular function than if these cells are functionally abnormal. The present study was designed to assess the capacity of granule cells generated in adulthood to participate in behaviorally-induced Arc expression in circuits of young and aged, memory-impaired F344 rats injected with BrdU 4 months earlier.

MATERIALS AND METHODS

Subjects

Twenty one Fisher344 rats were included in this study (Harlan Sprague Dawley, Indianapolis, IN). Eight rats were purchased at 5 months of age, while thirteen were purchased at 21 months of age. All animals were individually housed in an inverted 12 hr light cycle and maintained with water and food *ad libitum*.

BrdU Administration

All animals received daily injections of BrdU (50mg/kg i.p., Sigma) for 5 days. Afterwards, the animals were kept undisturbed in their home cages for 13 weeks. The rats were then handled for one week and tested in the Morris swim task (see below) for one week, and were then left undisturbed for one additional week prior to the final testing day. Thus, the total period from injection to tissue harvesting was sixteen weeks, and the age of animals at the time of tissue harvest was 9 and 25 months for young and aged animals, respectively. This delay interval was chosen for two major reasons. Because the response of newborn neurons may be fundamentally different in rats relative to mice (Snyder et al., 2009), it was important to investigate Arc expression in adult-generated neurons using a protocol that has previously been validated in rats (Ramirez-Amaya et al., 2006). Moreover, this length of delay between BrdU administration and behavioral Arc induction ensures that differences in

Arc expression are not due to a delayed or prolonged time course of neuronal maturation (i.e., Rao et al., 2005).

Behavioral Procedures

Thirteen weeks after BrdU administration, animals were handled for 7 days (15 min/day). After handling, the rats' spatial learning abilities were assessed using the Morris swim task (e.g., Morris et al., 1982; Shen and Barnes, 1996). Rats were given six spatial trials per day (24 trials total) with a hidden platform, and their location was tracked using a VP114 tracking unit (HVS image, England), and these data were used to calculate the rat's corrected integrated path length (CIPL), which is the sum of the rat's distances from the platform, corrected for the rat's initial distance and speed (Gallagher et al., 1993). One hour after the final spatial training trial, retention of the platform location was assessed using a probe trial in which the platform was removed. The probe trial was followed by 12 visual discrimination trials (6 the same day and 6 the following day) in which the platform was marked and clearly visible above the waterline. These trials ensure that age-related differences in spatial performance are not due to deficits in visual acuity or motor ability. Any aged animals failing the visible platform portion of the Morris swim task (i.e., performing outside the range of performance in young animals) were excluded from the experiment. Four aged animals were excluded on this basis, leaving 9 aged animals in the study.

Spatial Exploration

One week after training in the Morris swim task, 6 young and 6 aged animals underwent 5 min of spatial exploration, as described previously (Ramirez-Amaya et al., 2005). The exploration environment was a square open box, 61 × 61 cm with 20-cm-high walls, partitioned into nine grids. Each rat was placed in the center of one of the grids and moved to the center of a different grid every 15 s such that each of the grids was visited two or three times during the 5 min session. Immediately after the exploration session the animal was placed back in its home cage and kept undisturbed. The remaining 2 young and 3 aged rats remained in their home cages as control animals.

Tissue Preparation

Thirty minutes after the exploration session, animals were decapitated and the brains were extracted and frozen in 2-methylbutane (Sigma, St. Louis, MO) immersed in a slurry of dry ice and ethanol. The brains were stored at -70°C. Brain hemisections containing the dorsal hippocampus from eight to ten rats were molded in a block with Tissue-Tek OCT compound (Miles, Elkhart, IN), such that the block contained brains from all groups. The blocks were cryosectioned into 20µm coronal sections, thaw-mounted on superfrost plus slides (VWR), dried, and stored at -70°C before being shipped on dry ice to Wilfrid Laurier University for immunohistochemistry.

Immunohistochemistry

Twenty-five slides from the block were selected from the dorsal hippocampus for triple immunohistochemistry as previously described (Ramirez-Amaya et al., 2006). The tissue was fixed in 2% paraformaldehyde (pH 7.4, Sigma-Aldrich Canada, Oakville, ON) for 5 min, washed in TBS, and quenched with 1% H₂O₂ (in TBS) for 15 min. After blocking with tyramide signal amplification kit (TSA) blocking buffer (Perkin Elmer Canada, Woodbridge, ON), the slides were incubated (4°C for 24 hr) with biotinylated mouse-anti-NeuN (1:2000, Millipore), amplified by the ABC method (Vectastain ABC kit, Vector Labs Canada, Burlington ON), and labeled with Cy5 (TSA fluorescence system, Perkin Elmer). Following this treatment, the tissue was permeabilized with (1:1 v/v) acetone/methanol (1:1

v/v, Sigma) at 4°C for 5 min, and remaining biotin was blocked (Avidin/Biotin blocking kit, Vector). Slides were then incubated (4°C for 48 hr) in polyclonal rabbit anti-Arc (1:500, generously supplied by Dr. P.F. Worley, Johns Hopkins University), followed by biotinylated mouse anti-rabbit (Vector), and amplified by the ABC method (Vectastain ABC kit, Vector), and labeled with Cy3 (TSA fluorescence system, PerkinElmer). Following a blocking step for biotin (Avidin/Biotin blocking kit, Vector) and mouse IgG (MOM blocking kit, Vector), the DNA was denatured with a 50% formamide/2 × SSC (1:1 v/v, Sigma) at 65°C for 2h, incubated in 2N HCl at 37°C for 30 min, and washed in 0.1M borate buffer (pH 8.5, Sigma) for 10 min. before incubation (4°C for 24 hr) with mouse anti-BrdU (1:100, Roche) followed by biotinylated goat anti-mouse (Vector). Slides were then amplified by the ABC method (Vectastain ABC kit, Vector), and labeled with FITC (TSA fluorescence system, PerkinElmer).

Confocal Imaging and Analysis

Imaging and analysis was conducted as described previously (Ramirez-Amaya et al., 2006). Images were obtained with an Olympus FV1000 (Olympus Canada, Mississauga, ON) confocal microscope with using a 40× objective. Image stacks were collected from the whole thickness of the tissue (20µm), over the entire DG for each animal. The imaging parameters were set using the caged control on a given slide, and all images in that slide were taken using these parameters. At least 15 whole DG regions, taken from serial sections, were imaged for each animal. The most anterior section was taken ~5.5mm from the interaural plane.

Each DG was reconstructed with Photoshop (Adobe Systems Inc.) using the middle plane from each stack, resulting in a 2D image (Fig. 1). This image then was used as the reference image, and the 40× image stack was used to identify the BrdU⁺/NeuN⁺ and Arc⁺ cells. Each BrdU⁺ cell co-localizing with NeuN was considered a new neuron, and the Arc⁺ cells were considered the activated neurons. Each NeuN⁺ cell that was classified as BrdU⁺, Arc⁺, or both was annotated in the reference image. Duplicate cells found in adjacent image stacks were discarded to avoid overcounting. An average of 468.8 ± 58.7 cells were counted per blade in each animal's DG in this manner. The volume of the granule cell layer in which these cells were counted was then calculated in the reference image, as previously described (Ramirez-Amaya et al., 2005). The total number of granule cells within this volume of tissue was then estimated independently for young and aged animals by estimating the density of granule cells per cubic micron from 40× confocal image stacks, using the optical dissector, and extrapolating this estimate to the entire volume counted. The granule cell density estimate was derived from 110 z-stacks (65 from aged animals and 45 from young animals) collected at 40× magnification. No significant differences were observed in the density of granule cells between young and aged animals (data not shown), consistent with data that have been unable to detect a significant age-related loss of principle cells in any regions of the tri-synaptic circuit, including the DG, of the rat (e.g., Rapp and Gallagher, 1996; Rasmussen et al., 1996) mouse (e.g., Calhoun et al., 1998), non-human primate (e.g., West et al., 1993; Peters et al., 1996), or human (e.g., West, 1993). The proportion of granule cells expressing either Arc, BrdU, or both Arc and BrdU together were calculated using the estimated number of total granule cells. This estimate averaged $47,554 (\pm 3,042)$ cells per blade per animal. The proportion of mature new neurons expressing Arc represents the number of activated cells in the BrdU population while the proportion of Arc expressing cells in the already existing population represents the number of active cells in the non-BrdU population of granule cells.

Statistics

Spatial learning trials and visual trials in the Morris swim task were analyzed with repeated measures ANOVA, using training day as the repeated factor. Probe trial performance was assessed using one-way ANOVA. Analysis of *Arc* expression was conducted using a general factorial ANOVA with age, behavioral group (i.e., exploration vs. caged controls), and region (i.e., suprapyramidal vs. infrapyramidal blade) as factors. The probability of *Arc* expression in newborn vs. pre-existing granule cells was analyzed within each age group using a paired t-test in which the proportion of *Arc*⁺ neurons out of all BrdU⁺ neurons was paired with the proportion of *Arc*⁺ neurons among all BrdU-neurons within the same animal.

RESULTS

Aged animals show impaired spatial learning

Consistent with previous data (e.g., Gage et al., 1984; Gallagher et al., 1993; Rosenzweig et al., 1997; Shen et al., 1996), aged animals were significantly impaired in the spatial version of the Morris swim task (Fig. 2), but did not differ on the cued version of the task.

All rats showed behavioral improvement over trials (main effect of training day: $F_{3,48} = 8.12$; $p < 0.001$), but aged rats generally travelled longer paths to the platform than did younger rats (main effect of age: $F_{1,16} = 4.77$; $p = 0.04$). In addition, a significant interaction was observed between age and training day ($F_{1,16} = 4.53$; $p = 0.04$), suggesting that age-related differences in spatial learning grew progressively larger over the course of training. *Post-hoc* analyses confirmed that age-related differences in CIPL grew larger as training time progressed, reaching significance only on day 4 (day 1: $p = 0.25$; day 2: $p = 0.96$; day 3: $p = 0.06$; day 4: $p = 0.02$).

Similar age-related deficits in spatial memory were observed during the probe trial. Young rats spent significantly more time in the target quadrant of the pool that previously held the escape platform than did the old rats ($F_{1,16} = 14.4$; $p = 0.002$). These two groups of rats showed no difference, however, in the time spent in the maze quadrant opposite to the platform location ($F_{1,16} = 2.49$; $p = 0.13$).

However, during the visible platform version of the task, no significant difference was observed between the CIPL of young and aged animals (main effect of age: $F_{1,24} = 2.03$; $p = 0.18$), and no significant interaction was observed between age and training day ($F_{1,24} = 0.03$; $p = 0.88$). These data demonstrate that the aged rats in the present study had impaired spatial memory that is not accounted for by changes in visual acuity or motor abilities.

The number of new granule cells is reduced in aged animals

Comparable to previous reports (e.g., Kuhn et al., 1996; Bizon and Gallagher, 2003; Drapeau et al., 2003; Bizon et al., 2004; Montaron et al., 2006), the number of mature newborn granule cells observed in aged animals was significantly lower ($F_{1,26} = 180.41$; $p < 0.001$) than the number seen in young animals (Fig. 3a). Moreover, a comparable ($F_{1,26} = 0.10$; $p = 0.77$) decrease was seen in both the infrapyramidal and suprapyramidal blade. In addition, exploration did not influence the number of BrdU-labeled cells observed ($F_{1,26} = 1.832$; $p = 0.19$). This observation is consistent with the fact that BrdU injections (and thus incorporation) occurred 4 months prior to the exploration experience, long after the period in which exercise and/or spatial experience influences the proliferation and survival of newly-born granule cells (e.g., van Praag et al., 1999; Gould et al., 1999; Ambrogini et al., 2000).

Fewer granule cells express Arc in response to behavior in aged animals

Both young and aged animals respond to exploration by expressing Arc in significantly more granule cells relative to caged control animals ($F_{1,26} = 37.16$; $p < 0.001$). However, this behaviorally-induced Arc expression is blunted in aged animals (Fig. 3b). The overall proportion of granule cells in the young DG that respond to spatial exploration is 2.3% in the suprapyramidal blade (including both BrdU and non-BrdU cells). This number is significantly reduced in aged animals to 1.9% (main effect of age: $F_{1,26} = 5.80$; $p = 0.02$). In addition, this decrease is largely accounted for by BrdU⁻ cells. That is, the vast majority of Arc-expressing cells are not BrdU labeled, and a nearly identical pattern of significant decline from 2.2% in the young DG to 1.9% in the aged DG is observed if Arc⁺/BrdU⁻ cells are analyzed individually (main effect of age: $F_{1,26} = 5.54$; $p = 0.03$). Thus, the age-related decrease in the number of granule cells expressing Arc is accounted for largely by changes in the non-BrdU labeled population of neurons generated either before or after the availability of BrdU 4 months earlier.

Although Arc expression is generally far lower in the infrapyramidal blade (main effect of region: $F_{1,26} = 12.94$, $p = 0.001$), the number of granule cells expressing Arc is also reduced with progressive aging in this region (from 1.0% in young exploring animals to 0.8% in aged exploring animals) and thus no interaction is seen between age and region ($F_{1,26} = 0.22$; $p = 0.64$). Similarly, this pattern is nearly identical (1.0% in young exploring animals vs. 0.8% in aged exploring animals) if Arc⁺/BrdU⁻ cells are analyzed individually (main effect of region: $F_{1,26} = 12.74$, $p = 0.001$; age $\times$ region interaction: $F_{1,26} = 0.21$; $p = 0.65$).

Behaviorally-induced Arc expression in adult-generated (BrdU⁺) neurons is preserved with age

The overall proportion of cells that express Arc and are labeled for BrdU is significantly greater in the suprapyramidal blade than the infrapyramidal blade of the DG. Correspondingly (Fig. 3c), the number of cells that express Arc in the BrdU⁺ population is significantly greater in the suprapyramidal blade ($F_{1,26} = 4.43$; $p = 0.04$), as previously reported (Chawla et al., 2005; Ramirez-Amaya et al., 2006). Similarly, aged animals showed significantly fewer BrdU-labeled cells 4 months after injection, express Arc in fewer total granule cells, and had fewer BrdU⁺/Arc⁺ granule cells among the total population ($F_{1,26} = 14.10$; $p = 0.001$). The key question to be addressed in the current study, however, is not whether there is a significant decrease in the total number of cells co-expressing Arc and BrdU in aged animals. The fact that the aged DG produces so many fewer cells relative to the young DG ensures that this is the case. Rather, the key question is whether those cells that are generated and survive for several months in the aged DG remain capable of integration into existing neuronal networks. This question is directly relevant to whether the up-regulation of neurogenesis is a viable potential therapeutic target for treating age-related hippocampal dysfunction. This is addressed in the present study by examining the probability that BrdU-labeled cells in the aged DG will express Arc in response to spatial exploration behavior. An age-comparison of the fidelity of Arc expression can be made in both the BrdU-negative as well as BrdU-positive granule cell populations.

In the suprapyramidal blade, the overall proportion of granule cells in the DG of young animals that respond to spatial exploration is 2.3% (including both BrdU and non-BrdU cells). The BrdU-containing cells labeled over the course of 5 days of BrdU injections 4 months prior to sacrifice represent 0.7% of the population, while 99.3% were not labeled. When BrdU-expressing and non-BrdU-expressing granule cells are examined separately (Fig. 3d), 2.8% express Arc in response to the exploration treatment in the BrdU⁺ cell population of the suprapyramidal blade, significantly higher compared to 2.2% in the non-BrdU cell population ($t_5 = 2.39$; $p = 0.03$). Similarly, the proportion of BrdU-labeled cells in

the suprapyramidal blade of aged animals that express Arc in response to spatial exploration is significantly higher than in the remainder of the BrdU-granule cell population (2.9% vs. 1.9%; $t_5 = 3.29$; $p = 0.01$).

In the infrapyramidal blade, the overall proportion of DG granule cells in the DG of young animals that respond to spatial exploration is 1.0% and BrdU-containing cells represent 0.6% of the granule cell population. When BrdU-labeled and non-BrdU-labeled granule cells are examined separately (Fig. 3e), 1.4% express Arc in response to the exploration treatment, which although marginally higher, is not significantly different from the 1.0% of the non-BrdU cell population that express Arc ($t_5 = 1.59$; $p = 0.09$). The difference between blades is small, and thus inconclusive, however, this finding is consistent with a growing body of literature suggesting that the infrapyramidal blade of the DG typically shows attenuated activity relative to the suprapyramidal blade in response to spatial behavior (Chawla et al., 2005; Fletcher et al., 2006; Marrone et al., 2010b)

Thus, as previously reported (Ramirez-Amaya et al., 2006; Kee et al., 2007; but see Stone et al., 2010), mature, adult-born granule cells labeled with BrdU have a lower threshold for Arc expression relative to the remainder of the granule cell population. The current data extend these findings by showing that this effect holds true for both young and aged animals. There was no significant difference observed between the probabilities of BrdU-labeled granule cells in aged animals expressing Arc relative to non-BrdU-labeled cells that expressed Arc ($t_5 = 0.02$; $p = 0.98$). Moreover, when mature adult-generated granule cells were examined individually, the probability that an adult-born granule cell labeled with BrdU 4 months previously expressed Arc was equivalent in the young and aged DG across both the suprapyramidal and infrapyramidal blades (main effect of age: $F_{1,26} = 0.89$; $p = 0.77$; age $\times$ region interaction: $F_{1,26} = 1.14$; $p = 0.30$).

DISCUSSION

Consistent with previous data showing an age-related reduction in the number of granule cells that express Arc mRNA following exploratory behavior, the current study shows a concomitant decline in Arc protein. The truly novel finding in the present study, however, is that this reduction in the total number of granule cells that express Arc is not apparent in adult-generated (BrdU⁺) cells labeled 4 months earlier. Adult-generated cells of this age have an approximately equal probability of expressing Arc during behavior in both young and aged animals. Moreover, mature adult-generated granule cells continue to express Arc in response to spatial exploration in a significantly greater proportion than the remainder of the granule cell population for at least 4 months, in both age groups. On the basis of these data, it is tempting to conclude that information processing in the DG may be disrupted by a reduced number of adult-generated granule cells.

The finding of increased recruitment of adult-generated granule cells has received particular attention, and several hypotheses have been generated concerning the functional implications of this recruitment (Wiskott et al., 2006; Kempermann, 2008; Aimone et al., 2009; Alme et al., 2010). A recent experiment, however, has questioned the interpretation of these previous studies (Stone et al., 2010). In any comparison of the probability of recruitment in BrdU-labeled neurons relative to the total granule cell population, there is the potential for counting accuracy “bias”. For instance, counting cells that contain Arc among all NeuN-positive neurons creates the potential to underestimate the probability of recruitment of granule cells, since NeuN can be expressed in granule cells that are as young as a week old. Although the addition of very young NeuN-containing neurons would be a small number compared with the entire population of granule cells, these cells would not yet be capable of expressing Arc. To address this potential confound, Stone et al. (2010) directly

labeled granule cell populations at varying developmental points using distinct thymidine analogs. They found no preferential recruitment between developmentally- and adult-generated granule cells in mice up to 4 months of age. These apparently discrepant results may be due to fundamental differences in the behavior of newborn granule cells in rats compared with mice (Snyder et al., 2009), as the present data are comparable to those of a previous study in rats (Ramirez-Amaya et al., 2006). While the possibility remains that the present study has included immature granule cells not yet capable of expressing Arc in our counts, other differences in methodology have implications for the “underestimation hypothesis”. Foremost, Stone and colleagues have used animals of significantly younger ages than those at which the rats were sacrificed (~1 year old). At one year of age neurogenesis is dramatically reduced in all rodents relative to the first few months of life (e.g., Seki and Arai, 1995; Kuhn et al., 1996; Brunson et al., 2005), and thus there are substantially fewer immature granule cells within the unlabeled population available to inflate the estimate of Arc-negative granule cell numbers. This is particularly true for the aged animals, in which the rate of neurogenesis has decreased more than 5-fold. Moreover, at the long delay between BrdU labeling and testing in the present study (4 months) and in the Ramirez-Amaya et al. study (5 months), there is also the possibility of *overestimating* the probability of recruitment in the total granule cell population, due to the presence of granule cells born during the months after BrdU injection. Despite these differences, BrdU-labeled neurons are still found to be more likely to express Arc than the remainder of the granule cell population in these studies.

Regardless of the precise reasons for the differences observed between rat and mouse studies, these collective data clearly set serious limitations on the functional implications of granule cell recruitment in spatial learning. For instance, the preferential recruitment of newborn neurons has been proposed to allow the DG to temporally associate event memories that occur close together in time (Aimone et al., 2006; 2009). While it is not clear what the functional benefit would be of such temporal integration over a 4 month period – a time span that is nearly one-sixth of the total natural lifespan of a rat – it remains possible that this could provide some sort of temporal tag. It has also been suggested (Alme et al., 2010) that granule cells become progressively less responsive to stimulation with the passage of time, eventually reaching a point at which the majority of them are functionally silent in response to behavioral input. The current data are consistent with this notion. That is, there is a dramatic reduction in the generation of new granule cells in aged animals. Thus, the mean “age” of granule cells within the DG population increases dramatically during senescence. The reduction in the number of granule cells that express Arc as a result of behavior in aged animals may be explained by a greater proportion of granule cells being effectively ‘retired’.

The idea of granule cell retirement is also compatible with the observation made by Stone et al. (2010) that developmentally-generated and adult-generated neurons are equally likely to be active *when tested in 4 month old animals*. The data in Alme et al. (2010) and others (Sandoval et al., 2011) suggest that the probability of recruitment in an individual granule cell drops by approximately 50% by the age of 6–8 months. Moreover, the data from Alme et al. (2010) comes from estimates of activity in one context vs. several contexts, and so is also free of assumptions about the denominator (i.e., an estimate of total number of granule cells or their ability to express Arc). Collectively, these data are consistent with the idea that upregulating neurogenesis through manipulations such as physical exercise can create a neurogenic reserve that buffers age-related cognitive decline (Kempermann, 2008), and suggests that this reserve may be built by reducing the proportion of the granule cell population that is retired. Moreover, because adult-generated neurons in the aged DG retain the capacity to become integrated into behaviorally-relevant networks for at least 4 months,

it is plausible that interventions that rescue the relative rate of neurogenesis can rescue information processing long-term.

While the most efficacious intervention to up-regulate neurogenesis in aged mammals as well as the optimal time within the life course for these interventions remains to be determined, converging sets of data from rodent and human studies suggest that tasks that can measure pattern separation functions will be sensitive to DG manipulation. Pattern separation can be defined as the ability for the hippocampus to form uncorrelated representations of multiple, highly similar events or stimuli. Functional knockdown either of DG plasticity in general (e.g., McHugh et al., 2007; Niewoehner et al., 2007) or neurogenesis in particular (e.g., Dupret et al., 2008; Clelland et al., 2009) impairs the ability of animals to engage in tasks requiring discrimination of highly similar stimuli or locations. Moreover, a mounting body of evidence shows that aged animals (Dellu et al., 1992; Taghzouti et al., 1999; Marrone et al., 2010a) and humans (Bakker et al., 2008; Stark et al., 2010; Yassa et al., 2010) perform poorly in comparable tasks. These data are consistent with the theoretical role of the DG in pattern separation (e.g., Marr, 1971; McNaughton and Morris, 1987; McNaughton and Nadel, 1990; Treves and Rolls, 1992; O'Reilly and McClelland, 1994) and suggest that adult-generated neurons may be crucial for this process, consistent with some models for the functional role of neurogenesis (Aimone et al., 2009; Wiskott et al., 2006).

Concluding that granule cells generated during senescence are functionally normal, however, will require further investigation of both the abundance and consistency of behaviorally-induced Arc in newborn granule cells. The current data clearly show that newborn granule cells in aged DG are capable of expressing Arc in response to behavior. However, it remains unknown whether newborn cells generated in senescence produce comparable *amounts* of Arc protein to newborn granule cells in young animals. Penner et al. (2010) report that in the aged DG, there is a concomitant decrease in the abundance of *Arc* mRNA transcripts and an increase in methylation within both promoter and intragenic regions of the *Arc* gene. These data are consistent with the idea that the aged DG may be more susceptible to methylation-induced transcriptional repression. The effect of these changes on the abundance of Arc protein, however, remain to be determined, and may be complex given recent data showing that Arc translation can be dynamically regulated (Bramham et al., 2010; Greer et al., 2010). Arc protein expression in the DG following stimulation of the perforant path depends upon ERK-dependent signaling to MNK1, and, in turn, phosphorylation of eukaryotic initiation factor 4E (eIF4E; Panja et al., 2009). Moreover, the translation of *Arc* mRNA is regulated by nonsense-mediated decay that is dependent on eIF4AIII (Giorgi et al., 2007), and the stability of Arc protein is regulated by Ube3A (Greer et al., 2010). In young animals, data showing a tight coupling between *Arc* mRNA and Arc protein expression (Ramirez-Amaya et al., 2005) suggest that hippocampal Arc protein expression during behavior is regulated largely during transcription. Nevertheless, it remains possible that aging may alter these signaling events in a manner that may either further exacerbate or compensate for the decline in *Arc* mRNA production. Because changes in the relative abundance of Arc protein may have consequences on the long-term stability of activity patterns in granule cells, answers to these outstanding questions are crucial for understanding the impact of aging on this structure.

This issue is especially important considering that the behavior of adult-generated cells in response to multiple environments remains an area of considerable debate (Kee et al., 2007; Tashiro et al., 2007; Alme et al., 2010). Moreover, recent data suggest that the stability of activity patterns in multiple contexts may be particularly predictive of age-related memory decline. Several recent studies have shown that the capacity for granule cells to express immediate-early genes such as *Arc* both consistently upon repeated exposure to a single

environment (Small et al., 2004; Penner et al., 2010; Marrone et al., 2010a, b) and selectively in response to a single environment and not other, distinct environments (Marrone et al., 2010a) are particularly predictive of impaired spatial learning in aged animals. This leaves open the possibility that differences may yet emerge once age comparisons are made examining the responses of adult-generated cells to multiple contexts.

Despite these open questions, the observation that new neurons generated in senescence remain capable of functional integration satisfies the single largest requirement necessary for therapies that propose to stimulate neurogenesis for rescue of age-related cognitive decline.

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Figure 1.

Whole dentate gyrus (DG) reconstructions (scale bar = 200 μ m), assembled from 40 $\times$ overlapping confocal images from a young (**a, b**) and aged (**c, d**) animals that remained in the home cage (**a, c**) or explored a novel environment (**b, d**). Granule cells are labeled with NeuN and shown in blue (Cy5), Arc in red (Cy3), and BrdU in green (FITC). Note the reduction in BrdU-labeled cells (green) in aged animals. In addition, while the DG of both young and aged animals shows sparse expression of Arc protein predominantly in the suprapyramidal blade in response to spatial exploration, more activated cells are observed in the young DG (**d** vs. **b**). Higher magnification (Scale bar = 20 μ m) confocal projection

images show spatial exploration-induced Arc expression in a new granule neuron (BrdU⁺/NeuN⁺, white arrows) 4 months of age in both the young (**e**) and aged (**f**) DG. Only those cells that exhibited cytoplasmic Arc staining surrounding a BrdU-labeled nucleus were considered to be Arc-expressing BrdU neurons.

Figure 2.

Aged rats are impaired in the Morris swim task. Analysis of path lengths (**A**) shows that when the platform was hidden, young rats (black circle) swam shorter paths to reach the hidden platform than did the aged rats (black square), and this difference was significant on day 4 of training. During trials in which the platform was visible, young adult rats (open circle) and aged rats (open square) had comparable path lengths. During the probe trial (**B**), young adult rats (white) spent significantly more time than aged rats (grey) in the quadrant that previously held the platform (target), but not the opposite quadrant (all data are mean $\pm$ SEM; * $p < 0.05$; ** $p < 0.01$, young vs. aged animals).

Figure 3.

Arc expression in newly-born granule cells of the dentate gyrus (DG) is preserved with age. A dramatic decline occurs in the number of BrdU⁺ neurons (NeuN⁺) in aged animals relative to young ones (**a**). No significant differences were found in the suprapyramidal versus infrapyramidal blades of the DG. Significant differences in the percentage of Arc⁺ cells from the total population of granule cells (**b**) were found between young and aged exploring animals, although both young and aged exploring animals express Arc in more granule cells than caged controls. When the total percentage of cells co-expressing BrdU and Arc in the suprapyramidal and infrapyramidal blades of the DG are analyzed (**c**) a low, but statistically reliable, proportion of cells that showed both Arc and BrdU labeling in the young DG. This increase is not observed in granule cells of aged animals. However, when the proportion of Arc⁺ cells in the population of newly born (BrdU⁺) neurons is compared with the proportion of Arc-expressing cells in the population of unlabelled (BrdU⁻) neurons in the suprapyramidal blade of the DG (**d**), newly generated granule cells in both the young and aged DG are equally likely to express Arc in response to spatial exploration, and BrdU⁺ cells in both age groups are more likely to express Arc during exploration relative to the remainder of the granule cell population in both age groups. These effects are not observed (**e**) in the infrapyramidal blade (All data are mean $\pm$ SEM; *p < 0.05, **p < 0.01, young vs. aged animals within the same behavioral group; †p < 0.05, ††p < 0.01 relative to age-matched caged controls; ‡p < 0.05 paired t-test of the probability of Arc expression in BrdU⁺ vs BrdU⁻ cells within the same animal).