SYMPOSIUM REPORT

Newborn granule cells in the ageing dentate gyrus

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The dentate gyrus of the hippocampus generates neurons throughout life, but adult neurogenesis exhibits a marked age-dependent decline. Although the decrease in the rate of neurogenesis has been extensively documented in the ageing hippocampus, the specific characteristics of dentate granule cells born in such a continuously changing environment have received little attention. We have used retroviral labelling of neural progenitor cells of the adult mouse dentate gyrus to study morphological properties of neurons born at different ages. Dendritic spine density was measured to estimate glutamatergic afferent connectivity. Fully mature neurons born at the age of 2 months display ∼2.3 spines μm^{-1} and maintain their overall morphology and spine density in 1-year-old mice. Surprisingly, granule cells born in 10-month-old mice, at which time the rate of neurogenesis has decreased by ∼40-fold, reach a density of dendritic spines similar to that of neurons born in young adulthood. Therefore, in spite of the sharp decline in cell proliferation, differentiation and overall neuronal number, the ageing hippocampus presents a suitable environment for new surviving neurons to reach a high level of complexity, comparable to that of all other dentate granule cells.

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Normal ageing is accompanied by a general decline in cognitive, sensory and motor functions, and by numerous molecular, cellular, and structural changes (Mattson & Magnus, 2006). It has been proposed that alterations in a diversity of mechanisms of neuronal plasticity might underlie age-dependent deterioration in brain function (Burke & Barnes, 2006; Chawla & Barnes, 2007). In the dentate gyrus of the hippocampus the density of synaptic contacts formed onto granule cells is reduced by ageing (Geinisman *et al.* 1992). This change is also reflected by the decrease in the excitatory drive from perforant path axons impinging onto granule cells, and it is likely to underlie the decrease in behaviour-induced Arc expression in granule cells (Chawla & Barnes, 2007). This reduction in the input to the hippocampus is also accompanied by an altered plasticity of synaptic connections, since long-term potentiation displays a higher threshold and a weaker expression, whereas long-term depression is facilitated (Burke & Barnes, 2006).

Neurogenesis is another level of plasticity that is expressed in the dentate gyrus of the hippocampus through life, and it is also greatly affected by ageing. Neural progenitor cells of the subgranular zone divide, differentiate and migrate to produce a substantial amount of functional granule cells that become incorporated into the existing hippocampal circuitry (van Praag *et al.* 2002; Espósito *et al.* 2005; Ming & Song, 2005; Zhao *et al.* 2008). The number of newly generated neurons decreases sharply with age, due to a reduction in the rate of proliferation of neural progenitor cells (Kuhn *et al.* 1996; McDonald & Wojtowicz, 2005; Rao *et al.* 2005), in the number of neural progenitor cells (Olariu *et al.* 2007), and in the proportion of progenitor cells that adopt a neuronal phenotype (Kempermann *et al.* 1998; van Praag *et al.* 2005). Decreased neurogenesis has been proposed as a factor in the age-related decline of cognitive ability (Bizon & Gallagher, 2003; Drapeau *et al.* 2003; Kempermann *et al.* 2004). In fact, enriched environment and running increase neurogenesis in the senescent hippocampus and improve performance in the Morris water maze, suggesting that a reduction in adult hippocampal neurogenesis might be relevant to age-dependent cognitive decline (Kempermann *et al.* 1998; van Praag *et al.* 2005).

In contrast to the vast literature demonstrating the decreased number of new neurons in the hippocampus

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of ageing rodents, little is known about the specific characteristics of dentate granule cells that are born and survive in the normal ageing brain. Using retroviral fluorescent labelling and confocal microscopy we show that neurons born in the ageing dentate gyrus can develop typical granule cell morphology and spine density, suggesting that the mechanisms responsible for the reduction in neurogenesis might not affect the intrinsic neuronal properties of newborn granule cells.

Methods

A replication-deficient retroviral vector based on the Moloney murine leukaemia virus was used to express enhanced GFP driven by a CAG promoter (Laplagne *et al.* 2006). Female C57BL/6 mice were housed with or without a running wheel as described in the text. Retroviral injections were delivered to the right dentate gyrus at 2, 4, 6 or 10 months of age. Mice were anaesthetized (100 μg ketamine + 10 μg xylazine g^{-1}, I.M.) and surgery was performed as previously described (Laplagne *et al.* 2006). Animals were anaesthetized and perfused with paraformaldehyde at the indicated times. Housing, treatments, surgery and euthanasia were carried out according to NIH guidelines, and all experiments were carried out according to the guidelines laid down by the Leloir Institute animal welfare committee. Immunostaining was done on 40 μm free-floating coronal sections throughout the hippocampus. NeuN (mouse monoclonal, 1 : 50; a gift from F. H. Gage) and GFP (rabbit polyclonal, 1 : 100; Invitrogen) antibodies were combined, and corresponding secondary antibodies were used (donkey anti-mouse Cy5, donkey anti-rabbit Cy3, 1 : 250; Jackson ImmunoResearch, West Grove, PA, USA). Images were acquired using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). Three-dimensional reconstruction of dendritic segments was performed with the Zeiss LSM Image Browser Software from a series of 100–200 confocal planes taken at 0.1 μm intervals using a $\times$ 63 oil immersion objective. Spine density analysis was done by manually counting spines in dendritic fragments of ~400 μm for each of 5–7 GFP^+ cells per mouse, and 3–4 mice at each time point. Only GFP^+ dendrites located in the middle third of the molecular layer were included in the analysis. Neurons located in the upper and lower blade of the dentate gyrus were equally represented.

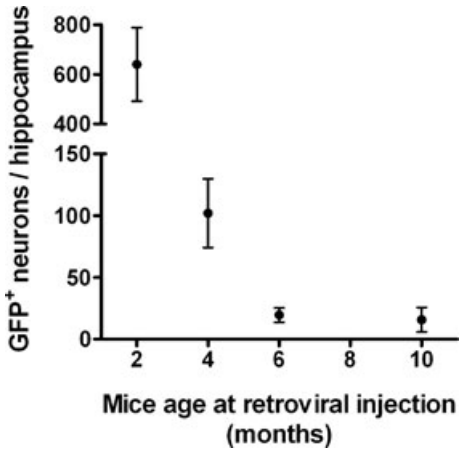

Figure 1. Age-dependent decline in adult neurogenesis
Mice were injected at the indicated time points. One every 3rd or 1 every 6th sections were used to count cells 4 weeks later to assess total number of GFP^+ granule cells per hippocampus. Data points represent mean ± S.E.M. obtained from 7, 5, 7 and 4 mice, for 2, 4, 6 and 10 months, respectively. The number of labelled neurons was significantly different at the age of 2 months compared to all other groups (ANOVA with $P < 0.0001$, followed by *post hoc* Tukey's test).

Results and Discussion

Sharp decrease in adult neurogenesis in the ageing brain

Bromodeoxyuridine (BrdU) labels nuclei of dividing cells and has been widely used as a marker for adult hippocampal neurogenesis. Previous BrdU studies have shown that neurogenesis decreases sharply in the ageing brain (Kuhn *et al.* 1996; Kempermann *et al.* 1998; McDonald & Wojtowicz, 2005; Rao *et al.* 2005; van Praag *et al.* 2005). Local retroviral labelling of adult-born neurons allows the study of their morphological and physiological features (van Praag *et al.* 2002; Ming & Song, 2005). To investigate whether the age-dependent decline in neurogenesis can also be observed with retrovirally labelled progenitor cells, a modified retrovirus expressing GFP was delivered to the dentate gyrus of mice at different ages. GFP^+ granule cells were counted 1 month later, after the critical period for neuronal survival had ended (Fig. 1). GFP^+ neurons were observed at all time points, although the number of newly generated neurons decreased significantly with age. A ~40-fold reduction was found when comparing 2- to 10-month-old mice, a sharper decrease than the one observed in previous BrdU studies that might be partially due to the limited effectiveness of retroviral labelling in the ageing brain (van Praag *et al.* 2005). This observation supports the notion that the mouse dentate gyrus has undergone substantial alterations throughout the ages studied in this experiment.

Complex morphological traits of neurons born in middle-aged mice

We have previously shown that neurons born in the hippocampus of young adult mice reach a degree of connectivity and function that is indistinguishable from neurons born during development (Laplagne *et al.* 2006,

2007). In this context, it is relevant to determine if neurons born in the ageing brain can achieve levels of function and complexity similar to neurons born in younger animals or whether this capacity is limited by age. In the young adult hippocampus, new granule cells achieve morphological and functional maturity within 6–8 weeks (Espósito *et al.* 2005; Piatti *et al.* 2006; Laplagne *et al.* 2006; Zhao *et al.* 2006). Accordingly, granule cells born in 2-month-old animals were analysed 2 months later and displayed a localization and overall dendritic and axonal morphology that are typical of mature dentate granule cells (Fig. 2*A* and *B*; '2 + 2' group). Similar anatomical properties were observed in neurons born at the same age but analysed 10 months later, in 1-year-old mice (Fig. 2*A* and *C*; '2 + 10' group), indicating that adult-born neurons can survive and maintain their basic structural complexity for several months.

To obtain a quantitative assessment of such long-term structural stability, dendritic spine density in the middle molecular layer was analysed for the different groups, since it provides an estimate of the density of glutamatergic synapses that will drive the excitatory activity of newborn neurons (although not all spines may reflect synapses with similar transmission efficacy). In addition, this parameter can be measured with high precision and repeatability and becomes independent of possible sectioning artifacts that might sever the dendritic tree. The same spine density (~2.3 spines μm^{-1}) was observed for dendritic

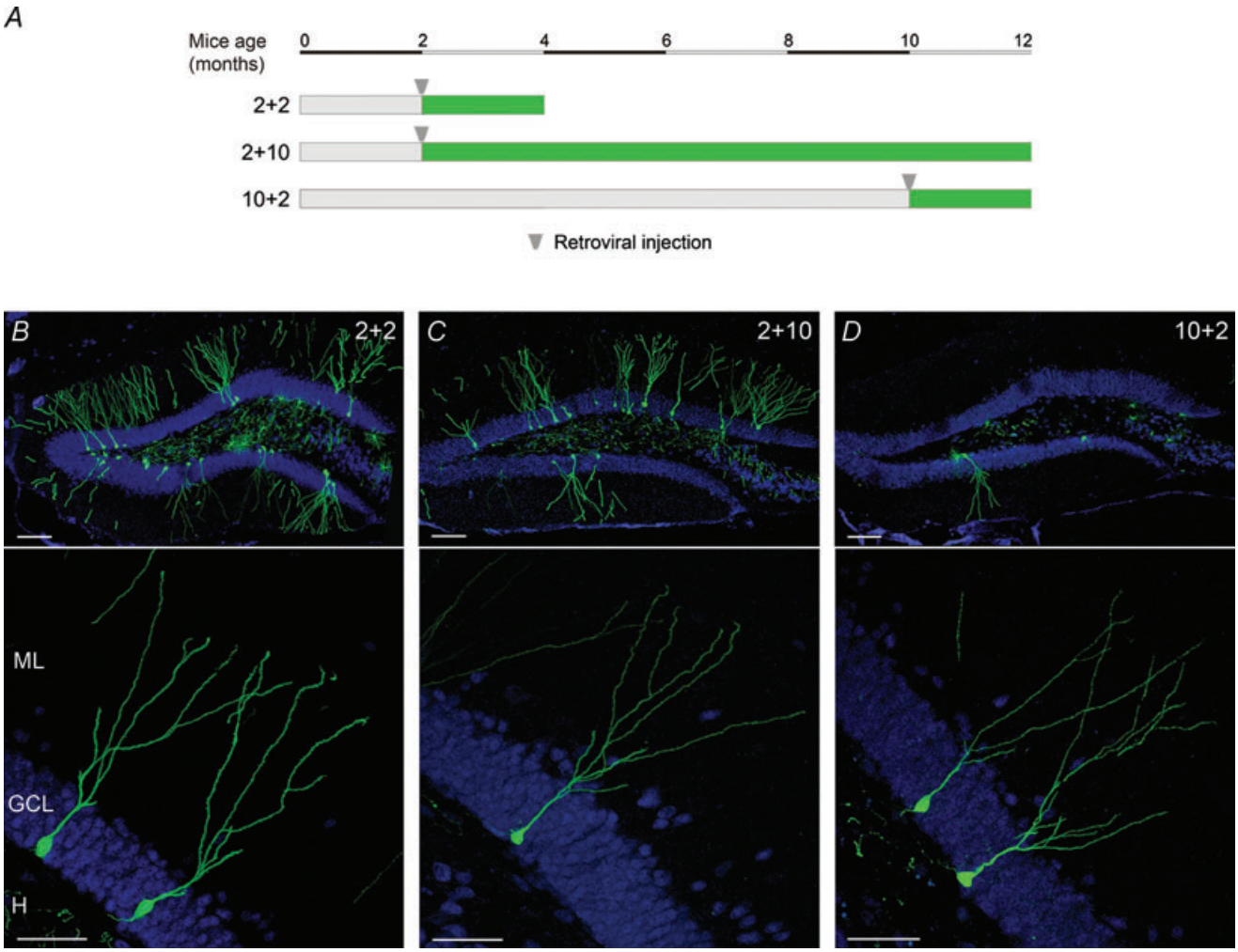

Figure 2. Morphological properties of neurons born in the young adult and ageing brain
A, experimental design denotes the age of retroviral injection (arrowhead), age of labelled neurons (filled bars), and the timing for data analysis (end of filled bar). The name of each experimental group is shown on the left. *B–D*, examples of confocal images showing an overview of the dentate gyrus for the different experimental groups (upper panels) and a more detailed view at higher magnification (lower panels). Images show GFP^+ cells in the context of the granule cell layer labelled with a NeuN antibody. Examples shown here are representative of 15–20 sections per mouse (2 + 2 group, $n = 4$ mice; 2 + 10 group, $n = 4$; 10 + 2 group, $n = 3$). Scale bars: 100 μm (upper panels) and 50 μm (lower panels). H, hilus; GCL, granule cell layer; ML, molecular layer.

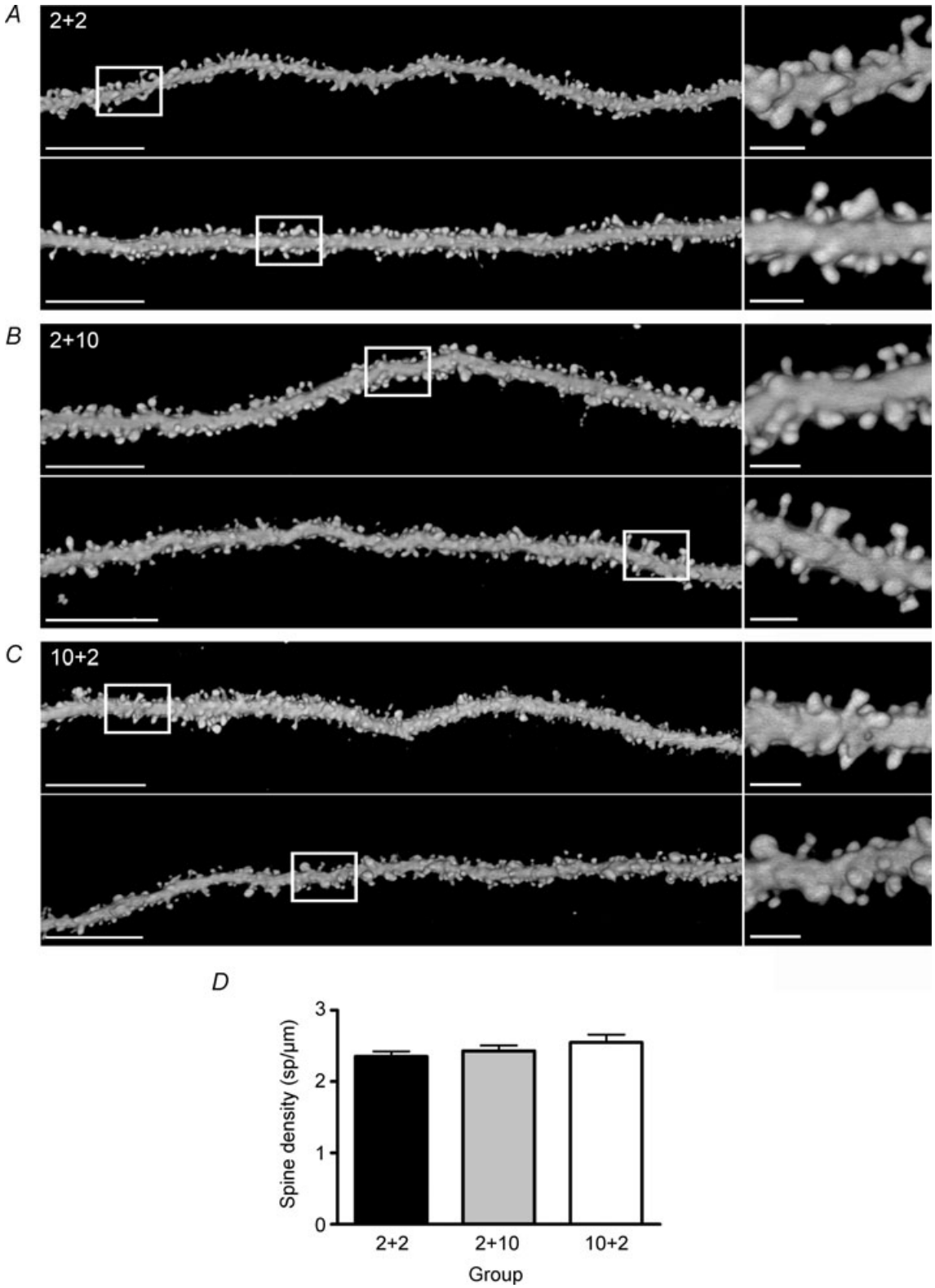

Figure 3. Dendritic spines of neurons born in the young adult and ageing brain
A–C, representative examples of spiny dendrites of newborn granule cells belonging to the 2 + 2 (*A*), 2 + 10 (*B*) and 10 + 2 (*C*) groups. Boxes on the left panels depict the area magnified in the right panels. Scale bars: 10 μm (left panels) and 2 μm (right panels). *D*, spine density measurements revealed no significant differences among the groups (ANOVA, $P = 0.28$). Spine counts were performed in total dendritic lengths of 1583 μm from 22 neurons (2 + 2 group), 1497 μm from 20 neurons (2 + 10 group) and 1333 μm from 19 neurons (10 + 2 group).

projections of neurons belonging to the 2 + 2 and 2 + 10 groups, suggesting that older neurons born in a young adult hippocampus preserve their afferent glutamatergic connectivity over time (Fig. 3*A*, *B* and *D*).

During this time the environment of the ageing brain does show progressive changes that are reflected by the decline in cognitive and learning abilities, synaptic plasticity and also neurogenesis (Fig. 1). In this context it is relevant to determine if neurons born in such an environment can develop, mature and connect normally, or if age-dependent factors restrict the degree of complexity that a newborn neuron can achieve. To address

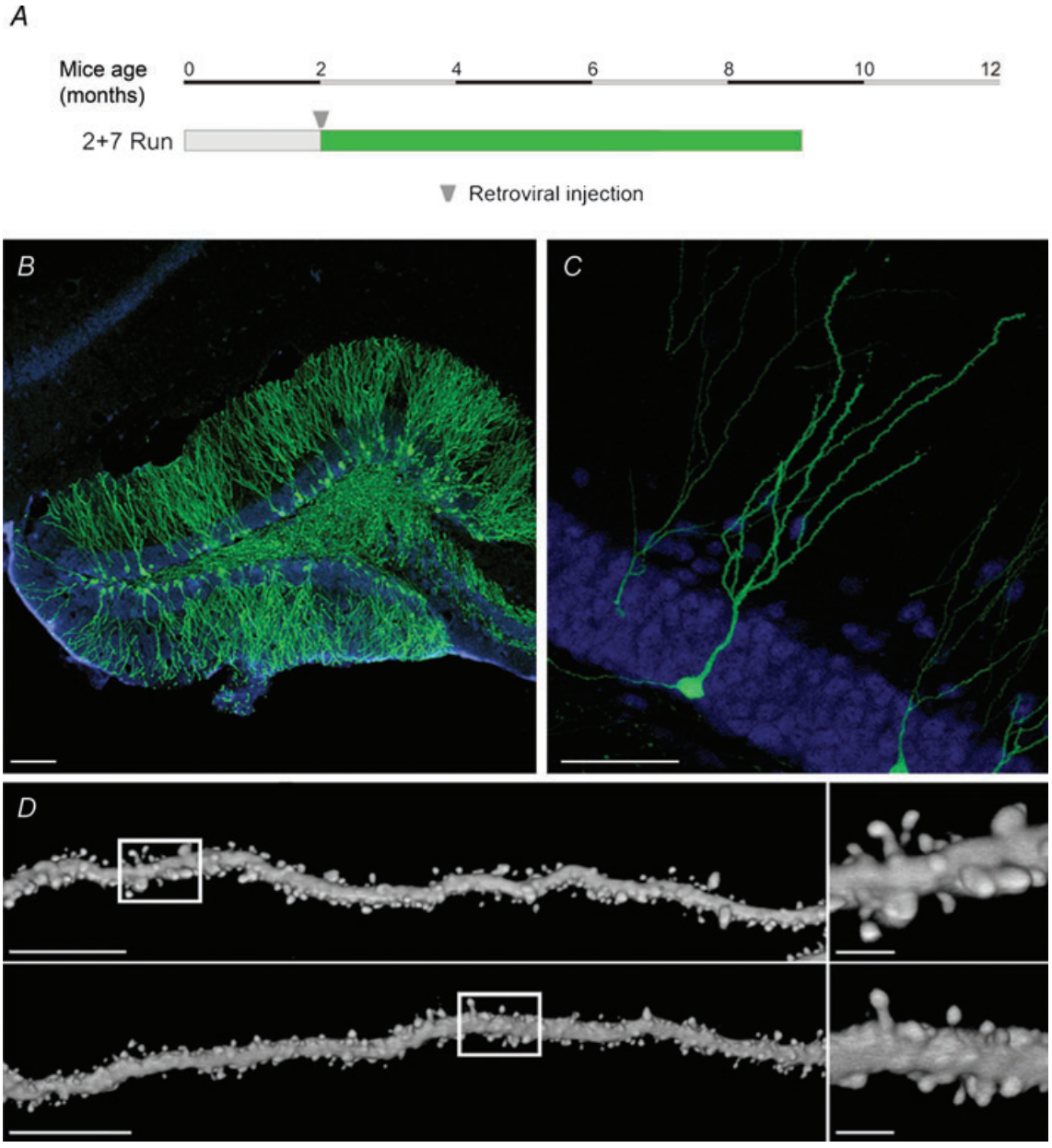

Figure 4. Newborn neurons in running mice
A, experimental design denotes the age of retroviral injection (arrowhead), age of labelled neurons (filled bars), the timing for data analysis (end of filled bar), and the name of the experimental group on the left. *B*–*C*, examples of confocal images showing an overview of the dentate gyrus (*B*) and a more detailed view at higher magnification (*C*). Images show GFP^+ cells in the context of the granule cell layer labelled with a NeuN antibody, and are representative of 15–20 sections per mouse from 4 runner mice analysed 7 months after retroviral injection. Scale bars: 100 μm (*B*), and 50 μm (*C*). *D*, representative examples of spiny dendrites of the 2 + 7 Run group. Boxes on the left panels depict the area magnified in the right panels. Analysis was performed on a total dendritic length of 1777 μm belonging to 25 neurons. Scale bars: 10 μm (left panel), 2 μm (right panel).

this question, dividing progenitor cells of the dentate gyrus were retrovirally labelled in 10-month-old mice and their neuronal progeny was analysed 2 months later (Fig. 2*A* and *D*; '10 + 2' group). As expected, this group showed a decreased number of GFP^+ neurons and an increased proportion of non-neuronal phenotypes such as GFP^+ glial cells (not shown). Surprisingly, newborn granule cells displayed a degree of structural complexity typical of mature granule cells born at a younger age. This similarity was also reflected in the number of glutamatergic synapses, since spine density in the 10 + 2 group showed no significant differences with either the 2 + 2 or the 2 + 10 groups (Fig. 3*C* and *D*). In this context, the reduced synaptic density reported in the ageing rat brain does not seem to be necessarily related to adult-born neurons, at least in middle-aged mice (Geinisman *et al.* 1992). Instead, such reduction might take place in pre-existing granule cells or become apparent at older ages.

Effects of running

Voluntary exercise such as running has been shown to increase the number of newborn neurons both in the young-adult and the old brain, as well as to accelerate the rate of dendritic spine maturation of new granule cells (van Praag *et al.* 1999, 2005; Zhao *et al.* 2006). In addition, exercise enhances performance in specific learning paradigms and ameliorates the ageing effects of certain neurodegenerative disorders (van Praag *et al.* 2000). In this context, the brain of a running mouse might be a more suitable environment for neuronal development and plasticity. To assess whether running exerted long-term effects in neurons developing in the adult hippocampus, granule cells born in 2-month-old mice housed with a running wheel were analysed 7 months later, at which time adult neurogenesis presents a striking decline. While the number of newly generated neurons was significantly increased, their basic morphology, distribution and complexity were comparable to all other groups shown above (Fig. 4*A*–*D*; '2 + 7 Run' group). Moreover, the observed spine density was 2.26 ± 0.07 spines μm^{-1} ($n = 25$ neurons, 4 mice), within the range of those values reported for non-runners in Fig. 3*D*. This is consistent with previous findings on young adult mice where running altered the kinetics of spine maturation without changing the plateau level for spine density (Zhao *et al.* 2006).

Concluding remarks

We have observed a remarkable similarity in the morphology of dentate granule cells born under conditions that can decrease (ageing) and increase (running) their number, and that are also known to exert strong modulatory effects in behaviour, synaptic plasticity, and neurodegeneration. Ageing substantially reduces proliferation and neuronal differentiation of neural progenitor cells, yet surviving neurons achieve a high degree of complexity with a density of afferent glutamatergic connections comparable to that of neurons born in young adult mice. Our findings suggest that surviving adult-born granule cells may be less sensitive than neural progenitor cells to environmental alterations of the ageing brain. Whether this robustness is reflected in the function of newly born granule cells needs to be further investigated, perhaps by combining electrophysiological approaches with studies on the activity-dependent expression of immediate early genes.

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