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# Aging brain microenvironment decreases hippocampal neurogenesis through Wnt-mediated survivin signaling

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## Summary

**Accumulating evidence suggests that adult hippocampal neurogenesis relies on the controlled and continued proliferation of neural progenitor cells (NPCs). With age, neurogenesis decreases through mechanisms that remain unclear but are believed to involve changes in the NPC microenvironment. Here, we provide evidence that NPC proliferation in the adult brain is in part regulated by astrocytes via Wnt signaling and that this cellular cross-talk is modified in the aging brain, leading to decreased proliferation of NPCs. Furthermore, we show that astrocytes regulate the NPC cell cycle by acting on the expression levels of survivin, a known mitotic regulator. Among cell cycle genes found down-regulated in aged NPCs, *survivin* was the only one that restored NPC proliferation in the aged brain. Our results provide a mechanism for the gradual loss of neurogenesis in the brain associated with aging and suggest that targeted modulation of survivin expression directly or through Wnt signaling could be used to stimulate adult neurogenesis.**

**Key words: adult neurogenesis; aging; astrocytes; survivin; Wnt signaling.**

## Introduction

The discovery of neural stem/progenitor cells in the adult central nervous system (CNS) has forever changed our view of the brain from a static tissue to one that is dynamic and adaptive. It has also created new expectations for the potential of cell therapy in the fields of aging and neurodegeneration. In mammals, adult neurogenesis occurs naturally in two discrete brain areas, the dentate gyrus (DG) of the hippocampus and the lateral ventricles (Alvarez-Buylla & Temple, 1998; Gage *et al.*, 1998). The hippocampus is of particular interest as its neuronal networks are involved in aspects of learning and memory. Studies have shown that hippocampal neural stem cells reside within the subgranular zone (SGZ). These cells give rise to neural progenitor cells (NPCs) that retain the ability to self-renew and have multi-lineage potential, differentiating preferentially into new granular neurons, but also into glia *in vivo* and *in vitro* (Alvarez-Buylla & Temple, 1998). The process of hippocampal neurogenesis encompasses proliferation of NPCs in the SGZ, survival and neuronal differentiation of newly born cells, migration of these newly differentiated neurons into the granule cell layer (GCL), and their functional maturation and integration into the synaptic circuitry of the GCL (Seri *et al.*, 2004). Newly generated neurons in the adult hippocampus are believed to play a crucial role in hippocampal-dependent learning and memory, a process commonly affected during the aging process. Indeed, hippocampal neurogenesis is typically robust in young adults but shows an exponential decrease with age in both rodents and primates (Kuhn *et al.*, 1996; Leuner *et al.*, 2007). The underlying molecular reasons for this age-related decline in hippocampal neurogenesis are currently not fully understood. The main difference between young and aged DG seems to be an age-dependent decrease in the rate of NPC proliferation rather than a change in NPC survival or neuronal differentiation (Rao *et al.*, 2005). Two processes could account for this reduction in proliferation in aged NPCs: a cell intrinsic alteration in the response to external mitogenic stimuli or a cell extrinsic change in the neurogenic niche. Intrinsic changes in NPCs with age are believed to occur, but need further investigation (Hattiangady & Shetty, 2008; Lugert *et al.*, 2010). By contrast, age-dependent changes in the neurogenic niche have been more widely reported and involve a decrease in positive regulators of neurogenesis (e.g., growth factors and cell cycle regulators; Hattiangady *et al.*, 2005; Shetty *et al.*, 2005; Molofsky *et al.*, 2006), with a concomitant increase in negative modulators (e.g., steroids and glutamate; Nacher *et al.*, 2003; Montaron *et al.*, 2006). The source of these signals remains to be precisely identified, but among the cellular components of the neurogenic niche, astrocytes are a primary candidate. This is not only owing to their number and physical proximity to NPCs but also because of their ability to instruct NPCs to adopt a neuronal fate (Song *et al.*, 2002). Indeed, astrocyte-mediated signaling is thought to play an important role in regulating the levels of neurogenesis (Lie *et al.*, 2005), yet further investigation is required to understand the mechanistic role of astrocytes during this process.

Here, we have investigated the cross-talk between astrocytes and NPCs in the context of the aging hippocampus, with a particular focus on its effects on NPC proliferation. We have identified the chromosomal passenger protein survivin (also known as *Birc5*) as a central intracellular mediator of age-associated changes in NPC proliferation, which we have correlated with the decrease in NPC proliferation during aging. Furthermore, our studies indicate that *survivin* regulation within NPCs is directly linked to a change in Wnt signaling initiated in the neurogenic niche by the astrocytes. Collectively, our data identify a mechanism that is involved in the age-dependent regulation of NPC proliferation by the neurogenic niche.

## Results

### Proliferation deficit of aged NPCs correlates with slower progression through mitosis

To better understand the age-related decline in NPC proliferation in the mouse DG, we determined whether the difference in young and old

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NPC proliferation could be replicated *in vitro*. Using *in vivo* bromodeoxyuridine (BrdU) incorporation in mice, we observed that by 13 months of age, the mouse DG showed a 10-fold reduction in BrdU-positive NPCs compared with 3-month-old mice (Fig. S1a,b). These two ages were therefore selected to isolate hippocampal NPCs (Ray & Gage, 2006). Basic microscopy analysis revealed morphological similarities among the two age groups of NPCs (Fig. S1c). Immunohistochemical analysis showed robust expression of the stem/progenitor markers, Sox2 and Nestin, in both NPC groups (Fig. S1d,f), suggesting that we were working with highly enriched NPC populations. In addition, like 3-month NPCs, 13-month NPCs retained the ability to differentiate into neurons and astrocytes *in vitro* (Fig. S1g). Both 3- and 13-month NPCs could be maintained in growth medium containing fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF). However, sufficient cells for assays were obtained in 6 weeks for 13-month NPCs compared with 4 weeks for 3-month NPCs, and 3-month NPC cultures required more frequent passaging compared with 13-month cultures, suggesting either increased cell death or decreased cell proliferation in 13-month NPCs. No differences in cell death were observed between 3- and 13-month NPC cultures, as assayed by annexin V and propidium iodide labeling (Fig. S1h). However, we observed differences in growth rates. At early passages (P1–P4), cultures of 13-month NPCs had significantly lower numbers of cells than similar cultures of 3-month NPCs (Fig. 1a), indicating a lower growth rate. These results were confirmed by quantification of the percentage of cells undergoing mitosis based on BrdU incorporation (Fig. 1b), expression of the pan-mitotic marker Ki-67 (Fig. 1c), or expression of the G2/M-specific marker phospho-histone H3 (pHH3; Fig. 1d). Furthermore, cell tracing studies indicated that all cells in the 13-month NPC cultures divided over the 4-day period (Fig. S1i). Therefore, differences in growth rates between 3- and 13-month NPC cultures were not likely due to the presence of a sub-population of slow or quiescent cells in the 13-month NPCs cultures, but most likely reflected an overall slower cell cycle progression in 13-month NPCs. To test this hypothesis, we evaluated the progression of cells through the G2/M phase of the cell cycle after nocodazole synchronization. As shown in Fig. 1e, nocodazole treatment leads to a 3- to 4-fold increase in the number of cells at the G2/M phase of the cell cycle. Upon nocodazole removal, differences in cell cycle progression were observed between 3- and 13-month NPCs (Fig. 1e,f). While ~90% of 3-month NPCs had completed the G2/M phase of the cell cycle by 12 h, only < 30% of 13-month NPCs transited through this cell cycle phase (Fig. 1f), suggesting a deficit in cell cycle progression.

## Proliferation of aged NPCs is restored after loss of exposure to the 13-month brain microenvironment

Despite the deficit in proliferation of 13-month NPCs initially, at later passages (P5 and later), their proliferation rate became indistinguishable from 3-month NPCs (Fig. 1a). Mitotic cell counts confirmed this result (Fig. 1b–d), indicating that the proliferation rate of 13-month NPCs increased in culture over time reaching levels similar to young NPCs. Within the same time period, the proliferation rate of 3-month NPCs did not change, suggesting that mitogens present in the medium did not induce cell transformation (Fig. 1a–d). To rule out gross genetic alterations in NPCs that could account for changes in proliferation rate, we cultured NPCs for 11 passages and performed cytogenetic analyses. No abnormalities were observed (Fig. S2). Furthermore, when cells were transplanted into immune-compromised athymic nude mice, we found no evidence of tumor formation over a 3-month observation period.

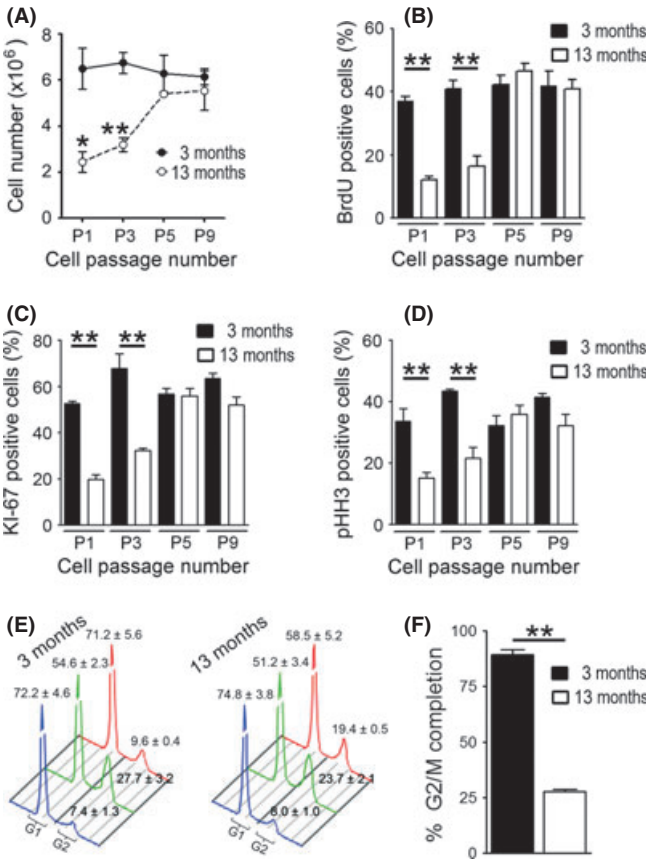


**Fig. 1** Proliferation deficit in aged neural progenitor cells (NPCs) is reversed with time in culture. (a) Total number of cells from 3- and 13-month NPCs cultures with time in culture. At each cell passage (P), cells were counted and replated at $1 \times 10^6$ cells per 10 cm dish. Cells were passaged every 4 days. (b–d) Levels of cell proliferation observed in 3- and 13-month NPCs with time in culture as determined by the percentage of cells positive for the S-phase mitotic marker BrdU (b), the pan-cell cycle marker KI-67 (c), or the G2/M cell cycle marker pHH3 (d). (e) Cell cycle profiles of 3- and 13-month NPCs under proliferative conditions (blue), following nocodazole treatment (green), and after release from nocodazole block (red). (f) Percentage of cells that have completed the G2/M phase of the cell cycle as show in (e). Mean values ± SEM from triplicate experiments are shown; *$P < 0.05$; **$P < 0.01$.

## Molecular analysis of the effects of aging on the cell cycle of NPCs

To identify a molecular mechanism for the reduced proliferation of 13-month NPCs at early passages in culture, we conducted a detailed quantitative gene expression analysis by profiling over 100 genes involved in cell cycle regulation. Among them, nine genes were reduced by greater than twofold in 13-month compared with 3-month NPCs (Fig. 2a and Table S1). These nine genes functionally regulate multiple cell cycle stages including cell cycle transition, cell cycle checkpoints, and cell cycle arrest. In total, our gene array and marker analysis suggests that the proliferation deficit of 13-month NPCs is owing to an overall decrease in the number of cells in the active phase of the cell cycle and could involve repression of a subset of genes that regulate cell cycle progression.

## Survivin rescues the proliferation deficit of 13-month NPCs in culture

To determine whether decreased expression of any of the nine genes identified could account *per se* for the observed proliferation defect in

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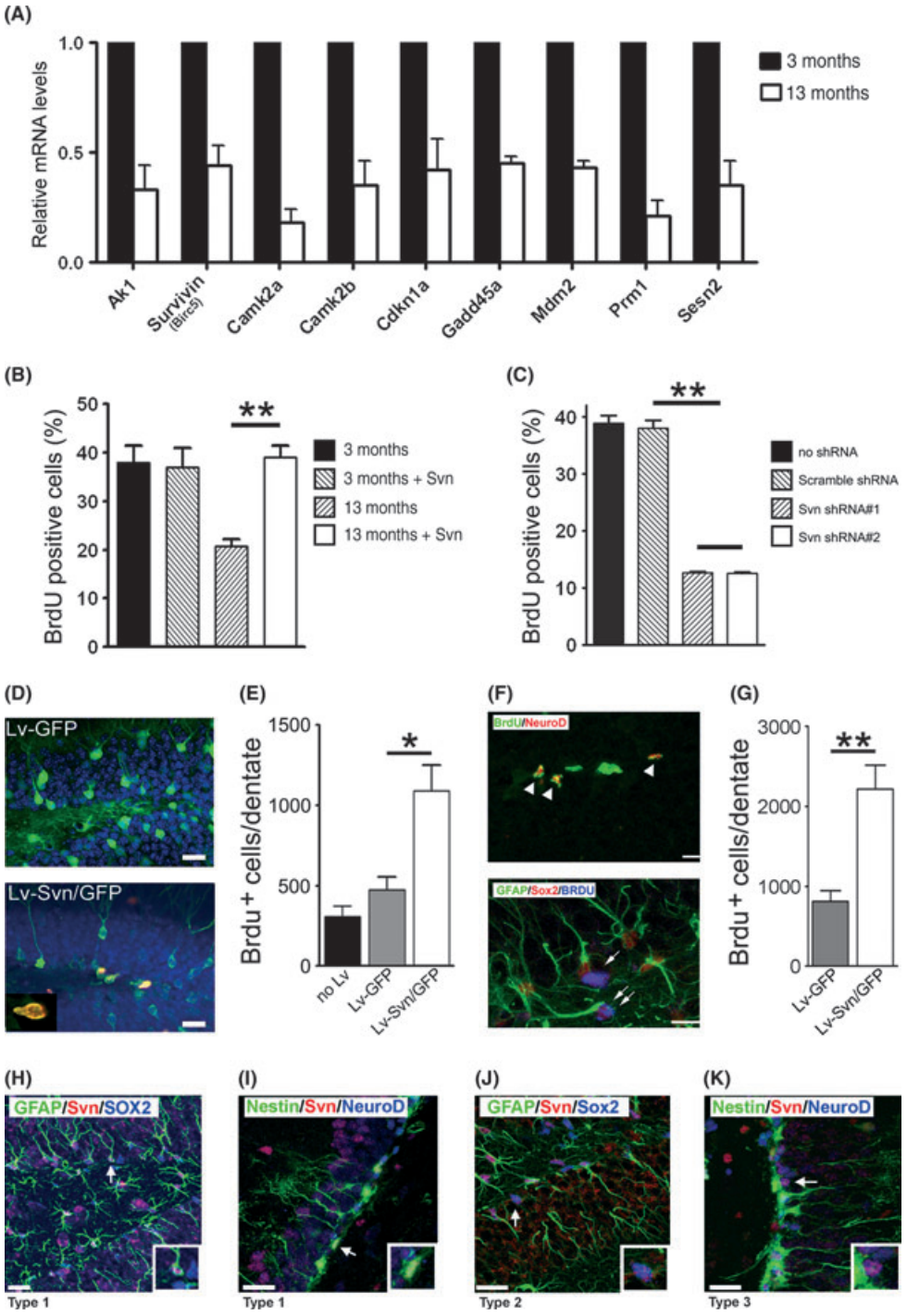


**Fig. 2** Survivin levels in neural progenitor cells (NPCs) are decreased with aging and regulate proliferation. (a) Cell cycle regulatory genes down-regulated in 13-month compared with 3-month NPCs as determined by qRT-PCR. Gene analysis was performed P2. (b) Effects of exogenous survivin expression on the proliferation of 3- and 13-month NPCs (P3). (c) Effects of survivin knockdown on the proliferation of 3-month NPCs. (d) Representative image showing transduction of cells with lentivirus expressing GFP (Lv-GFP) or *survivin*-GFP (Lv-Svn/GFP) in the granular layer and subgranular zone (SGZ). $BrdU^+$ cells were found only in the SGZ and co-localized with GFP-expressing cells. (e) Quantification of BrdU+ cells in the dentate gyrus (DG) of control, Lv-GFP injected, or Lv-Svn/GFP injected 13-month mice during a daily 7-day BrdU pulse. $N = 6$ mice per group. (f) Identity of BrdU+ cells transduced with Lv-Svn/GFP. Type 1 (double arrow), type 2 (single arrow), and type 3 (arrowheads) NPCs are indicated. (g) Quantification of BrdU+ cells in the DG of Lv-GFP or Lv-Svn/GFP injected 13-month mice during a 24-h BrdU pulse. Survivin expression can be found in Sox2+, GFAP+, Nestin+, NeuroD type 1 NPCs (h–i) Sox2+, GFAP type 2 NPCs (j) Nestin–, NeuroD+ type 3 NPCs (k). Cells identified by the arrows in h–k are shown magnified in the accompanied figure inset. In all graphs, mean ± SEM from triplicates are shown, $*P < 0.05$. $**P < 0.01$. Scale bars, 20 μm.

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13-month NPCs, cells were transiently transfected with constitutively active expression constructs encoding cDNAs for each gene. Despite the high levels of transfection efficiency (60–70%), none of the nine genes increased the proliferation rate of 3-month NPCs above control (Fig. S3a). By contrast, among all nine genes, only the chromosomal passaging protein, survivin, was able to rescue the proliferation deficit of 13-month NPCs and increased their proliferation rate to levels comparable with 3-month NPCs (Figs 2b and S3b). Of note, survivin has been shown to be predominantly expressed in the G2/M phase of the cell cycle (Li *et al.*, 1998), in agreement with our finding that 13-month NPCs progress slower through G2/M than 3-month NPCs (Fig. 1e,f). In support of a correlation between survivin expression levels and NPC proliferation rates, we found that both survivin mRNA and protein levels increased in 13-month NPCs at later times in culture (Fig. S3c,d) just as their proliferation rate also gradually increased (Fig. 1a–d). Furthermore, reduction in survivin protein levels in 3-month NPCs using shRNAs resulted in a significant reduction in the proliferation rate and survival of 3-month NPCs despite the continuous presence of pro-mitotic factors in the culture medium (Fig. 2c). A similar decrease in proliferation was obtained with 3-month NPCs isolated from mice with a genomic deletion of one *survivin* allele (Jiang *et al.*, 2005; Fig. S3e).

### Sustained survivin expression in NPCs augment neurogenesis in the aged hippocampus

We next sought to evaluate whether ectopic survivin expression in hippocampal NPCs could overcome the natural age-related decline in NPC proliferation *in vivo*. We accomplished this by targeting slowly dividing NPCs in 13-month-old mice. Stereotaxic injection of lentivirus expressing a *survivin*-green fluorescent protein fusion construct (Lv-Svn/GFP) into the right side of the DG, resulted in an approximate 2.5-fold increase in the number of BrdU-positive labeled cells compared with mice injected with a control GFP-only lentivirus (Fig. 2d,e). Indeed, the majority of BrdU-positive cells (~93%) also expressed the survivin–GFP fusion protein (Fig. 2d). Marker analysis performed in brain sections, where the GFP signal was quenched, revealed that the majority of BrdU-positive cells in 13-month Lv-Svn/GFP mice expressed NeuroD1, a marker of type 2/3 NPCs (Gao *et al.*, 2009; Fig. 2f top). In addition, a few BrdU-positive cells were positive for GFAP and Sox2 or Sox2 alone that identify less committed type 1 and type 2 NPCs, respectively (Fig. 2f bottom). Of note, the levels of NPC proliferation observed in the 13-month brain were still significantly lower compared with the 3-month brain (Fig. S3f), most likely due to the low transduction of type 1 and 2 NPCs. To more stringently assess any confounding effects of survivin on NPC survival over the 7-day period, we performed a shorter 24-h BrdU pulse in mice transduced with Lv-Svn/GFP. A similar 3-fold increase in BrdU-positive NPCs in 13-month Lv-Svn/GFP-transduced hippocampus was observed with this shorter pulse (Fig. 2g), indicating that this increase in NPC numbers is solely owing to an effect of survivin on NPC proliferation. Of note, when the same Lv-Svn/GFP lentivirus was stereotaxically injected in 3-month-old mice, no significant increase in proliferation was observed (Fig. S3f), suggesting that unlike other trophic factors, survivin does not act as a general inducer of NPC proliferation or cell survival.

### Survivin is expressed in all NPC subtypes of the DG

Adult DG NPCs reside in the SGZ and are believed to be unique astrocytes expressing GFAP and exhibiting radial glia properties (Morrens *et al.*, 2012). To determine whether survivin may be implicated in cell cycle regulation of all NPCs or only a restricted subset, we analyzed *in vivo* co-localization of survivin with markers that identify NPCs at different stages. Using a combination of antibodies (GFAP, Sox2, and NeuroD) and Nestin–GFP reporter mice, we found that survivin was expressed in type 1 cells identified by expression of GFAP, Sox2 (Fig. 2h), and Nestin (Fig. 2i). Survivin was also detected in type 2 cells that are positive for Sox2, but lack GFAP expression (Fig. 2j) and in type 3 cells that are negative for the marker, Nestin, but positive for the marker NeuroD1 (Fig. 2k). Survivin expression could also be detected in granular neurons, albeit at much reduced levels compared with NPCs (Fig. S4). In conclusion, the finding that survivin is expressed in all NPC subtypes suggests that decreased survivin levels with age may affect all NPCs and may globally impact adult DG neurogenesis.

### Survivin is required for maintenance and proliferation of adult NPCs, but not for their differentiated progeny

To determine whether survivin is specifically required for the proliferation of NPCs or has an additional effect on the progeny of differentiated NPCs, we isolated NPCs from *survivin* floxed mice ($svn^{L/L}$ mice; Jiang *et al.*, 2005). Neural progenitor cells *survivin* nulls obtained after Cre expression showed a significant reduction in cell viability compared with control, Cre-transduced wild-type NPCs (Fig. 3a,b). Morphological analysis of *survivin* null NPCs showed an increased number of binucleated cells (Fig. 3c). Cell cycle analyses revealed a G2/M arrest (Fig. 3d), in agreement with a role for survivin in chromatid segregation during mitosis (Mita *et al.*, 2008; Fig. 3e). Survivin deficiency in NPCs ultimately led to apoptosis (Fig. 3f), likely due to an inability to complete the cell cycle. Overexpression of potent anti-apoptotic proteins could not rescue the *survivin* null NPCs from apoptotic cell death (Fig. S5a,b). To evaluate the requirement for survivin expression in NPCs during hippocampal neurogenesis, retroviral vectors, which expressed a GFP/Cre construct (Tashiro *et al.*, 2006) and were designed for long-term gene expression *in vivo,* were stereotaxically injected into the DG of $svn^{L/L}$ mice (Fig. 4a), thus targeting dividing NPCs (mostly type 2/3) residing in the SGZ. Control $svn^{L/L}$ or wild-type mice received an injection of retrovirus encoding GFP alone or GFP/Cre, respectively. Sixty hours after injection, we evaluated the total number of GFP-positive cells in injected animals and found no differences in the number of GFP-positive cells in $svn^{L/L}$ mice or GFP/Cre wild-type treated animals. We next evaluated GFP expression at 3 weeks postinjection, a time frame during which newborn cells normally integrate into the hippocampal circuitry (Zhao *et al.*, 2008). In control animals, we found large numbers of GFP-expressing cells that had incorporated into the DG as newborn neurons (Fig. 4b,c), with projections to the CA3 region of the hippocampus (Zhao *et al.*, 2008). By contrast, no GFP-positive cells were present in $svn^{L/L}$ animals treated with Rv-GFP/Cre (Fig. 4b,c), suggesting that the targeted NPC cells perished owing to the lack of survivin. In contrast, ablation of *survivin* in neurons and astrocytes both *in vivo* and *in vitro* had no deleterious effects on the survival of these cells (Fig. S6a–f), suggesting that survivin is not functioning as a general pro-survival factor in neurons and astrocytes.

### NPC *survivin* promoter activity is modulated by factors within the neurogenic niche

Our initial observation that the proliferation rate and survivin expression increases over time in cultured 13-month NPCs (Figs 1a–d and S3c,d) suggested to us that environmental factors rather than a cell autonomous intrinsic aging program could be responsible for the observed proliferation deficit of NPCs in the aging brain. To directly demonstrate that survivin expression is modulated *in vivo* by environmental factors and that this

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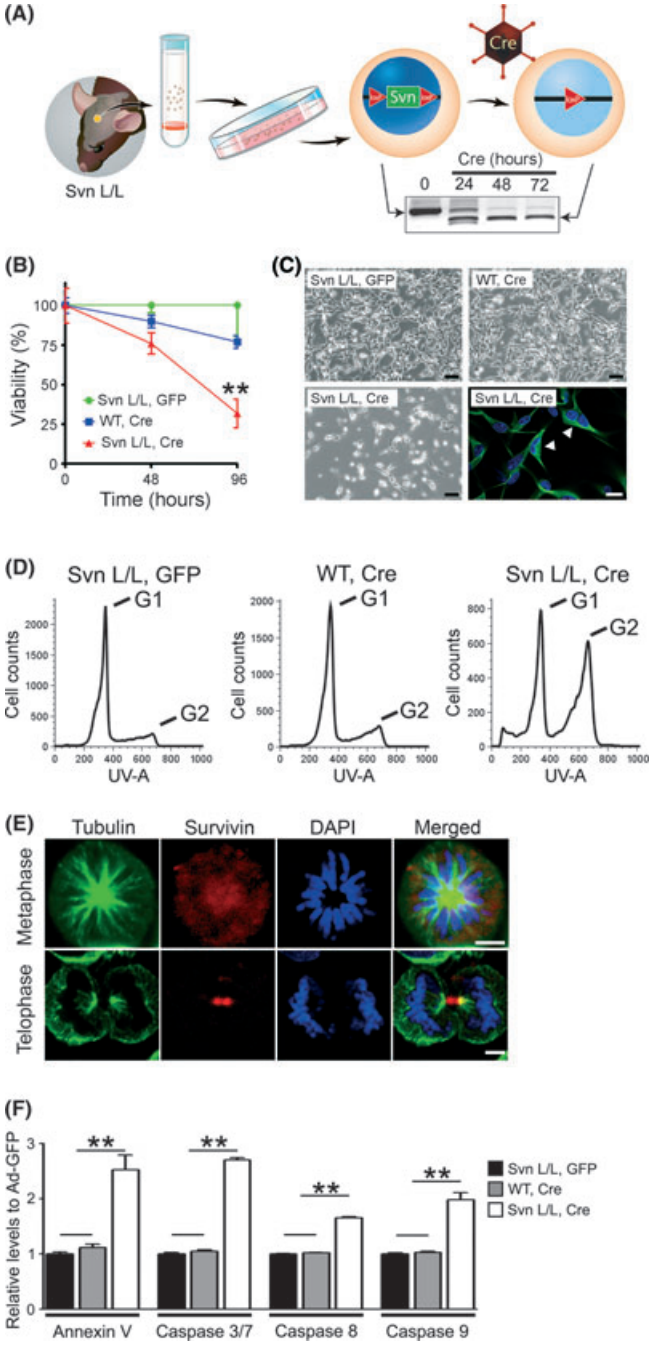


**Fig. 3** Survivin is required for the proliferation and survival of neural progenitor cells (NPCs). (a) A schematic of the experimental procedure used to ablate *survivin* in NPCs. *Survivin* was deleted using adenoviral-mediated expression of Cre-recombinase. (b) Cell viability was determined by the MTT assay in survivin expressing NPCs ($svn^{L/L}$ – GFP, Green and wild-type–Cre, Blue) and survivin-deficient NPCs ($svn^{L/L}$ – Cre, Red). (c) Phase contrast and fluorescence images showing decreased cell viability and presence of cells with two nuclei in survivin-deficient NPCs (bottom panel) isolated from $svn^{L/L}$ mice 72 h post Ad-Cre infection compared with Ad-GFP in $svn^{L/L}$ or Ad-Cre in wild-type NPCs (upper panel). (d) Cell cycle profiles as obtained by flow cytometry analysis of DNA content of proliferating $svn^{L/L}$ NPCs treated with Cre showing arrest in G2/M compared with wild-type treated controls. (e) Intracellular localization of endogenous survivin in NPCs during mitosis. Scale bars, 5 μm. (f) Levels of annexin V positive cells and caspase activation found in $svn^{L/L}$ NPCs culture 72 h post Cre induction. In all graphs, mean values ± SEM from triplicates are shown, $^{**}P < 0.01$.

modulation is age-dependent, the hippocampus of 3- and 13-month-old mice was stereotaxically injected with either 3- or 13-month NPCs transduced with a dual reporter system that drives expression of GFP under a constitutive promoter and red fluorescent protein (RFP) under a *survivin* promoter. This system allowed reliable detection of all transplanted cells (GFP positive) and simultaneous evaluation of *survivin* promoter activity (RFP expression levels). Strikingly, transplantation of 13-month NPCs into the hippocampus of 3-month-old mice resulted in high levels of RFP expression, indicating that upon exposure to a young brain environment *survivin* transcription is activated in 13-month NPCs (Fig. 5a). Conversely, a 40–70% reduction in RFP expression levels, and therefore *survivin* promoter activity, was observed in 3- or 13-month NPCs, respectively, when transplanted into the hippocampus of 13-month-old animals (Fig. 5a,b). These results suggest that the 13-month DG either expresses factors that repress *survivin* promoter activity or lacks factors that positively regulate *survivin* promoter activity. Taken together, these data strongly suggest that *survivin* promoter activity in NPCs can be regulated by components of the neurogenic microenvironment and that this regulation is modified with age.

## Factors secreted by astrocytes regulate NPC survivin levels and proliferation in an age-dependent fashion

As astrocytes have been shown to instruct neuronal differentiation of adult NPCs (Song *et al.*, 2002), we therefore asked whether astrocytes may also modulate adult NPC proliferation in an age-dependent manner. To test this hypothesis, primary astrocytes isolated from 3- and 13-month-old mouse brains were maintained in culture to obtain conditioned medium. Both 3- and 13-month NPCs, engineered to express luciferase under control of the *survivin* promoter, were exposed to astrocyte-conditioned medium to quantify effects on NPC *survivin* expression levels and on proliferation rates. Conditioned medium from 3-month astrocytes increased *survivin* promoter activity and proliferation in both 3- and 13-month NPCs compared with base medium alone (Fig. 5c,d). By contrast, conditioned medium from 13-month astrocytes decreased *survivin* promoter activity by 50% in both 3- and 13-month NPCs compared with base medium alone (Fig. 5c). This was accompanied by a significant decrease in NPC proliferation (Fig. 5d). These results mirror our *in vivo* observations following transplantation of NPCs into the hippocampus of 3- or 13-month-old mice (Fig. 5a). Furthermore, these data suggest that astrocytes secrete both activators and inhibitors of *survivin* promoter activity thus directly affecting the rate of NPC proliferation. In addition, our data indicate that these astrocyte-secreted factors change with age in a manner consistent with the observed age-related decline in NPC proliferation.

## Astrocyte-secreted Wnts regulate *survivin* expression and proliferation of adult NPCs

Based on recent evidence that Wnt/β-catenin signaling regulates NPC and stem cell proliferation and differentiation (Lie *et al.*, 2005; Kalani *et al.*, 2008), we sought to determine whether astrocytes utilize this pathway to regulate NPC survivin expression. Activation of the canonical Wnt pathway stabilizes β-catenin, which subsequently forms a transcriptionally active complex with members of the TCF/LEF transcription factor family. To monitor activation of this canonical pathway, 3- and 13-month NPCs were stably transduced with a TCF/LEF reporter construct. Media supplemented with recombinant Wnt 1, 3, and 3a (hereafter referred as Wnts) led to activation of the TCF/LEF reporter in NPCs (Fig. S7a), and this response could be inhibited by addition of a specific Wnt inhibitor,

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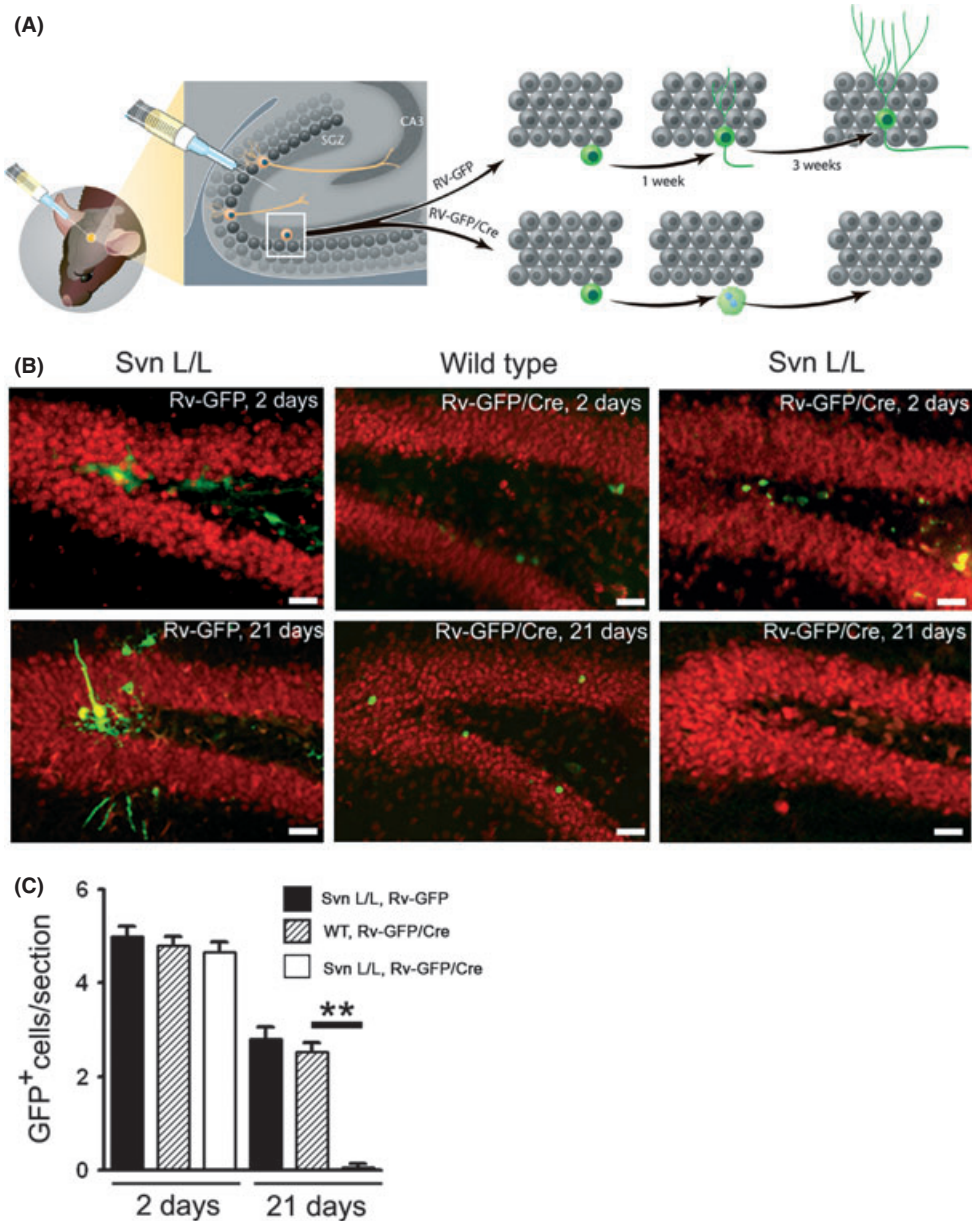


**Fig. 4** Creation of newborn adult hippocampal neurons requires survivin expression in neural progenitor cells (NPCs). (a) Schematic of the experimental approach depicting dividing NPCs in the subgranular zone transduced with retroviruses (Rv) carrying GFP (cytoplasmic localization) or a Cre-GFP fusion protein (nuclear localization). Hippocampi were analyzed 3 weeks later for the presence of GFP+ granular neurons recognized by NeuN expression. (b) Similar numbers of NPCs were transduced with Rv-GFP or Rv-GFP/Cre and expressed GFP 2 days post injection into wild-type or $svn^{L/L}$ mice (upper panels). After 3 weeks (bottom panels), approximately 50% of cells initially transduced with Rv-GFP in $svn^{L/L}$ mice or with Rv-GFP/Cre in wild-type mice were still present in the granular layer and express NeuN. By contrast, in Rv-GFP/Cre injected $svn^{L/L}$ mice, no GFP-transduced cells were observed at 3 weeks (bottom middle panel). (c) Quantification of GFP-positive cells found following Rv-GFP and Rv-GFP/Cre at day 2 and 21 as in 'b'. $^{**}P < 0.001$, $n = 8$ for each time point). Mean values ± SEM are shown.

soluble Frizzled Receptor Proteins 2/3 (sFRP 2/3). These results indicate that NPCs do not lose responsiveness to Wnt/β-catenin signaling with aging. Interestingly, activation of the canonical Wnt signaling pathway led to an increase in survivin protein levels in 13-month NPCs (Fig. S3d), in agreement with studies in cancer cells where survivin is a transcriptional target of TCF/LEF (Tapia *et al.*, 2006). Therefore, both 3- and 13-month NPCs respond to Wnts, and the activation of the canonical Wnt pathway leads to *survivin* induction.

We next asked whether astrocytes release Wnts that activate *TCF/LEF* in 3-month NPCs. Conditioned medium from 3-month-old astrocytes increased *TCF/LEF* promoter activity in 3-month NPCs compared with base medium alone, and this activation could be further enhanced by exogenous addition of Wnts (Fig. S7b). By contrast, conditioned medium from 13-month astrocytes reduced *TCF/LEF* promoter activity over baseline, indicating both the presence of Wnt inhibitors and the lack of proper Wnt levels in 13-month astrocyte-conditioned medium. Addition of Wnts to conditioned medium from 13-month astrocytes significantly activated the *TCF/LEF* promoter indicating that NPCs were still able to respond to Wnts (Fig. S7b) and suggests that Wnt expression in astrocytes decreases with age. To test this hypothesis, we examined the relative expression lev-

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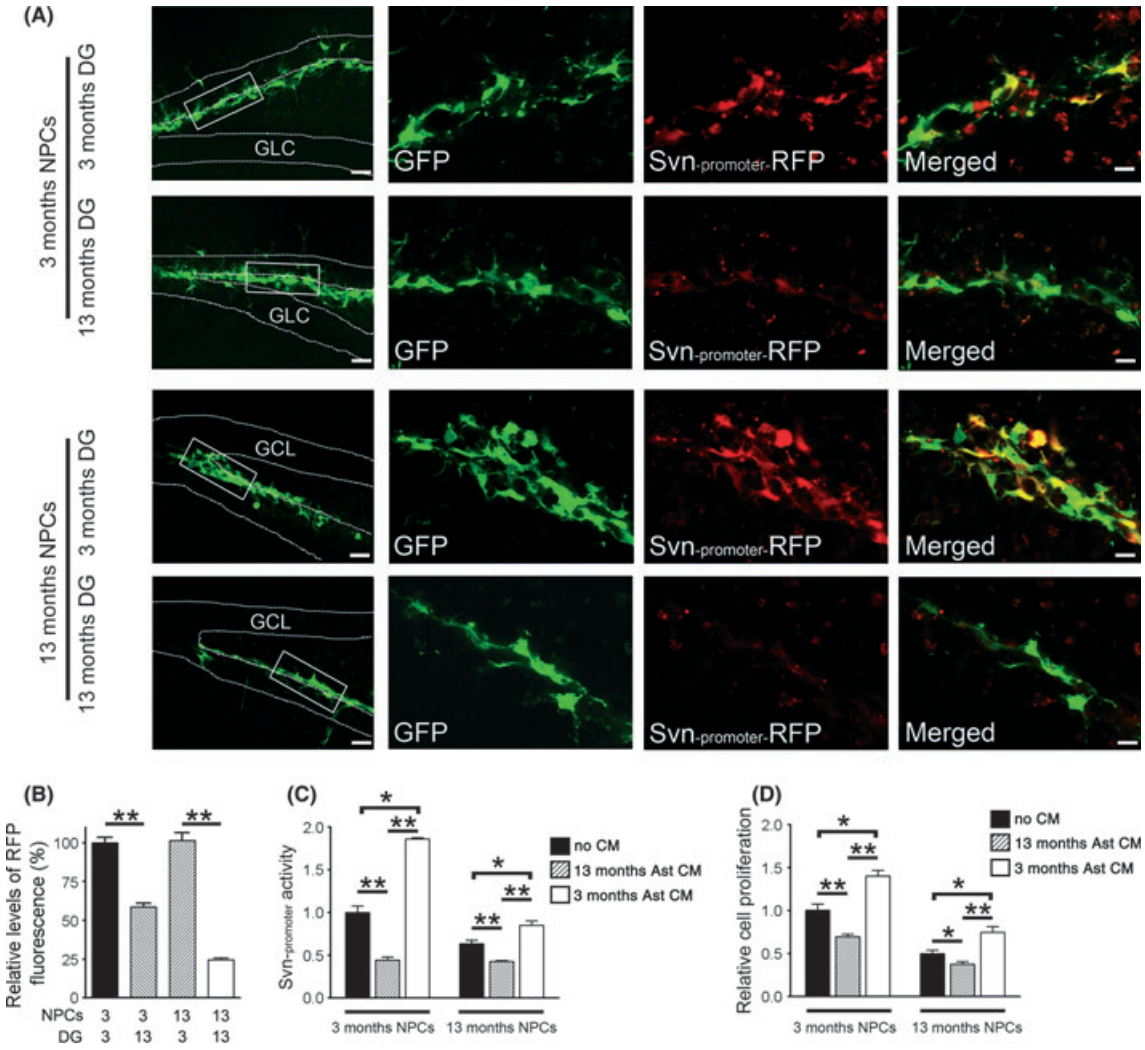


**Fig. 5** The neurogenic microenvironment of older mice represses neural progenitor cell (NPC) *survivin* expression and NPC proliferation. (a) *Survivin* promoter activity (red) in 3- and 13-month GFP$^+$ NPCs (green) transplanted into the dentate gyrus (DG) of 3- or 13-month-old mouse brains. Representative images ($n$ = 6 mice) are shown for each experimental group. (b) Quantification of red fluorescence levels in transplanted NPC cells visualized in 'a'. Bottom numbers indicate age in months of transplanted NPCs and recipient mouse DG. (c, d) Effect of conditioned medium (CM) from 3- or 13-month astrocytes on *survivin* promoter activity (c; firefly luciferase reporter activity) and proliferation (d; BrdU incorporation) in 3- and 13-month NPCs. Mean percentages ± SEM from triplicate experiments are shown. *$P$ < 0.05; **$P$ < 0.01. Scale bars, 20 μm.

els of 17 known *Wnt* isoforms in astrocytes isolated from 3- and 13-month-old mice and found that most *Wnts* were down-regulated in the older astrocytes (Fig. 6a). We next assessed whether Wnts released by astrocytes are responsible for the observed effects of astrocyte-conditioned medium on survivin levels and ultimately NPC proliferation. Strikingly, inhibition of Wnt signaling by sFRP2/3 nearly abolished the increase in *survivin* promoter activity induced by 3-month astrocyte-conditioned medium in NPCs, even in the presence of exogenous Wnts (Fig. 6b), strongly suggesting that survivin up-regulation by astrocytes is mediated by Wnt signaling. Accordingly, conditioned medium from 13-month astrocytes supplemented with recombinant Wnt proteins showed a significant increase in *survivin* promoter activity in NPCs that was abolished by Wnt inhibition (Fig. 6b). Furthermore, inhibition of Wnt signaling in the presence of 3- or 13-month-conditioned medium supplemented with Wnts was accompanied by a significant reduction in NPC proliferation (Fig. 6c). Of note, addition of sFRP2/3 did not fully inhibit the proliferative effects of 3-month astrocytes-conditioned medium on NPCs, indicating that other factors present in 3-month astrocyte-conditioned medium contribute to the increase in NPC proliferation (Fig. 6c). To further confirm that Wnts induce NPC proliferation via survivin, we down-regulated survivin in 3- and 13-month NPCs using two independent shRNAs and found that these cells were unable to proliferate in response to Wnts (Fig. 6d). Taken together, these results demonstrate that Wnts released by astrocytes promote NPC proliferation by inducing survivin expression and that Wnt production by astrocytes decreases with aging.

## Discussion

Adult NPCs reside in a specific microenvironment, the neurogenic niche that provides signals needed to maintain and control proliferation and instruct precursor differentiation (Palmer *et al.*, 2000). Many studies have shown a decrease in neurogenesis with aging and have proposed a link to changes in the neurogenic niche (Garcia *et al.*, 2004; Luo *et al.*, 2006). The age-related decline in neurogenesis can be caused by either a deficit in proliferation or in the differentiation potential of NPC. Studies have implicated Wnts released by astrocytes in the neuronal differentiation of

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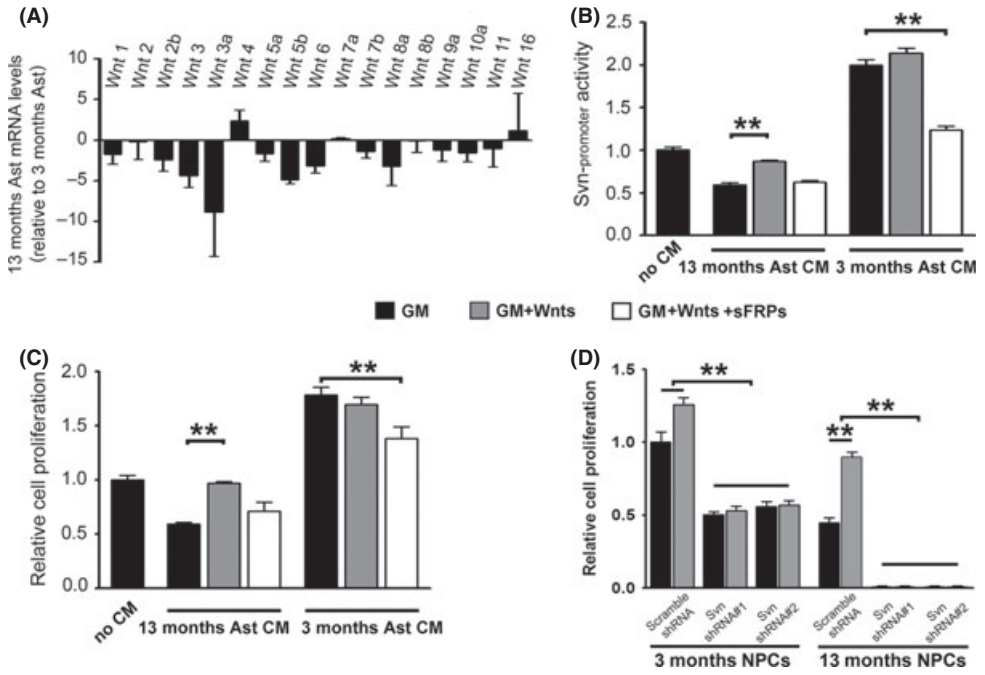


**Fig. 6** Age-dependent reduction in astrocyte-mediated Wnt signaling correlates with a decrease in survivin levels and proliferation of neural progenitor cells (NPCs). (a) Quantitative RT-PCR comparison of *Wnt* family gene expression levels between 3- and 13-month astrocytes (Ast). (b, c) Effects of Wnts and Wnt inhibitors (sFRPs) on *survivin* promoter activity (b) and proliferation (c) in 3-month NPCs in the presence of conditioned medium (CM) from 3- or 13-month astrocytes (Ast). Growth medium (GM) contains fibroblast growth factor 2 and epidermal growth factor. (d) Survivin knockdown abolishes Wnt effect in augmenting NPC proliferation. Mean values ± SEM from triplicates are shown. $^{**}P < 0.01$.

adult NPCs (Lie *et al.*, 2005) and recently was shown that Wnt production by astrocytes, specifically Wnt3, decreases with aging and correlates with decreased neurogenic differentiation of NPCs in the aged brain (Okamoto *et al.*, 2011). Here, we show that Wnt secretion by astrocytes is also implicated in the second aspect of the age-related decline in neurogenesis, namely the decline in NPC proliferation. Furthermore, we have not only identified the trigger within the neurogenic niche but also an effector gene within NPCs that mediates the changes in their proliferation: survivin. In addition, we provide evidence that increased expression of survivin in NPCs *in vitro* and *in vivo* can counteract the age-related decline in NPC proliferation. Our data support a model in which a reduction in astrocyte Wnt-mediated signaling that occurs with aging in DG leads to a down-regulation of *Survivin* expression in NPCs and therefore to the quiescence of NPCs in aged CNS. Further genetic studies are needed to determine whether additional mechanisms also act on survivin expression, including regulation of promoter methylation status (Esteve *et al.*, 2005).

These results highlight the pivotal role of astrocytes in brain function and together with recent reports, identify astrocytes as a key component of the adult neurogenic niche (Song *et al.*, 2002; Lie *et al.*, 2005). These observations are consistent with the current view that adult brain astrocytes, in addition to providing structural support for neurons, also perform numerous functions that include forming neuronal-glial-vascular units, which regulate the blood–brain barrier and defend the nervous system against a multitude of pathological insults. In addition, astrocytes can also participate in determining the progression and outcome of neuropathological processes, as shown in various neurodegenerative diseases, including amyotrophic lateral sclerosis and Rett's syndrome, and therefore should be considered important therapeutic targets (Haidet-Phillips *et al.*, 2011; Lioy *et al.*, 2011).

In the hippocampus, astrocytes reside in close proximity to NPCs, suggesting that they interact actively with developing neurons within the adult neurogenic niche (Seri *et al.*, 2004). The finding that these cells both instruct neuronal fate and promote adult NPC proliferation (Song *et al.*, 2002) supports a functional role for hippocampal astrocytes in adult neurogenesis. To date, understanding this process has been difficult owing to the lack of identification of a molecular mechanism by which astrocytes control NPC proliferation. Here, we report that the control of Wnt signaling within NPCs is one of the mechanisms that becomes altered during aging. Secreted Wnt molecules have been shown to be important not only for normal brain development but also for postnatal neurogenesis by regulating NPC self-renewal, proliferation, and differentiation (Lie *et al.*, 2005; Kalani *et al.*, 2008). The findings that most Wnts are down-regulated in aged astrocytes and that this signaling is required for NPC proliferation strengthens the hypothesis that the reduction in hippocampal neurogenesis during the aging process is associated with the quiescence of NPCs (Hattiangady & Shetty, 2008; Lugert *et al.*, 2010; Bonaguidi *et al.*, 2011); however, they may also be depleted (Encinas *et al.*, 2011). Furthermore, our results indicate that age-related NPC quiescence can be reversed by exposure to Wnts or survivin upregulation, pointing to potential therapeutic opportunities for conditions where hippocampal neurogenesis is suppressed. They provide two candidate targets: modulation of survivin within NPCs and regulation of Wnt levels in the neurogenic niche (Foust *et al.*, 2010).

In summary, these results demonstrate that by middle age, significant changes have occurred in the neurogenic niche resulting in decreased NPC proliferation. They provide further evidence for a noncell autonomous regulation of neurogenesis in aging and provide in part a molecular understanding of pathways that control the decline in NPC proliferation in aging. Further studies identifying additional regulators within the

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neurogenic niche controlling the function of NPCs are needed to extend our knowledge of the molecular mechanisms underlying reduction in DG neurogenesis with aging.

## Experimental procedures

A more comprehensive description of methods associated with this work can be found in the online supplemental information accompanying this article.

### Mice

All procedures were performed in accordance with the NIH Guidelines and approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital. C57BL/6 obtained from Jackson Laboratories (Bar Harbor, ME, USA) were bred and aged in our facility. Survivin floxed ($Svn^{L/L}$; Jiang *et al.*, 2005) and Nestin-GFP (Lagace *et al.*, 2007) reporter mice have been described.

### BrdU injections

Both 3- and 13-month mice received either one or ten intraperitoneal injections of Bromodeoxyuridine (Sigma-Aldrich, St Louis, MO, USA) at the dose of 50 mg $kg^{-1}$ body weight for 7 consecutive days or during 24-h period, respectively.

### Isolation and culture of neural progenitor cells

NPCs were isolated from the hippocampus of 3- and 13-month C57BL/6 mice as previously described (Ray & Gage, 2006). Two weeks after isolation, large floating neurospheres were visible. Spheres were transferred to polyornithine-/laminin-coated (P-/L-coated) 10-$cm^2$ plates where they grew as a monolayer in proliferative medium composed of DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing N2 supplement (Invitrogen), FGF-2 (20 ng $mL^{-1}$; Peprotech, Rocky Hill, NJ, USA), EGF (20 ng $mL^{-1}$; Peprotech), and heparin (5 μg $mL^{-1}$; Sigma-Aldrich, Ray & Gage, 2006). Cells were passaged when reaching confluency of 70%, considered as passage 1 (P1). All assays were performed after first passage (3–4 weeks for 3 month and 4–6 weeks for 13 month from cell isolation).

### Immunofluorescence staining of cultured NPC and brain sections

Paraformaldehyde-fixed cells or brains were incubated for 1 h at room temperature in TBS + 0.1% triton-X and 10% donkey serum. Samples were incubated 24–48 h at 4 °C in primary antibodies, followed by secondary antibodies. All images were captured on a Zeiss confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

### RNA isolation and quantitative RT-PCR

Total RNA was isolated (Qiagen, Germantown, MD, USA), and Quantitative RT-PCR arrays for cell cycle and Wnt signaling pathways were obtained from SABiosciences (Qiagen).

### Cell transfection

cDNAs were obtained from Origene (Rockville, MD, USA). Amaxa transfections were performed (Lonza, Walkersville, MD, USA) with transfection efficiency determined by GFP levels to be 60–70%.

### Viral vectors

Adenovirus-GFP and adenovirus-Cre were obtained from Vector Biolabs (Philadelphia, PA, USA) and used at MOI of 1:100. Retrovirus expressing GFP and GFP/Cre were a generous gift from Dr. Gage and described previously (Tashiro *et al.*, 2006). Lentiviral vectors coding the sequence of Survivin or the survivin promoter sequence were prepared in our laboratory. DNA vectors used to clone Survivin cDNA sequence (EGFP expressed in-frame with survivin and the *survivin* promoter into viral vectors have been previously described; Otaki *et al.*, 2000; Caldas *et al.*, 2005). Viral particles were concentrated to $1 \times 10^9$ infection units per mL by ultracentrifugation (Tiscornia *et al.*, 2006).

### Stereotaxic injections

Brain coordinates were −2.0 anterior–posterior, ± 1.5 mediolateral, and −1.75 dorsoventral to target the hippocampus (DG) and +0.5 anterior–posterior, ± 2.0 mediolateral, and −2.5 dorsoventral to target striatum. To evaluate survivin expression in neurons, AAV9-GFP or AAV9-GFP/Cre ($1 \times 10^9$ infection units, 1 μL), was injected in 8–12 weeks wild-type and $Svn^{L/L}$ mice. To evaluate the effect of survivin overexpression in promoting NPCs in 3- and 13-month DG, lentiviral vectors expressing GFP or Survivin/GFP ($1 \times 10^9$ particles per mL; 2 μL) were injected into the right hippocampus of C57Bl/6 mice.

### Nocodazole treatment and cell cycle analysis

For analysis of Nocodazol treated cells, 3- and 13-month NPCs were cultured in proliferation medium composed of DMEM/F12 supplemented with N2 growth factors. For cell synchronization at the G2/M transition, cells were incubated with 600 ng $mL^{-1}$ of nocodazole (Sigma-Aldrich) for 15 h. Cells were obtained every 2 h and stained with Hoechst 33342. Cell fluorescence was measured by flow cytometry.

### Cell transplantations

To track transplanted cells, 3- and 13-month NPCs were transduced with a ubiquitous green fluorescent protein (Lv-CAG-GFP). Next, cells were transduced with a lentivirus encoding the RFP under control of the *survivin* promoter. Transplanted NPCs were identified by GFP expression. Survivin promoter activity was assayed by RFP intensity and evaluated 72 h post-transplantation.

### Primary astrocyte culture

Astrocyte cultures from the hippocampus of 3- and 13-month C57BL/6 mice were prepared as previously described (Noble & Mayer-Proschel, 1998).

### Luciferase assays

TCF/LEF activation assays were performed in NPCs transfected with the TOPFlash plasmid that contains TCF-/LEF-binding sites (Addgene, plasmid #12456, Cambridge, MA, USA). Luciferase assays were performed using the Bright-glo luciferase assay (Promega, Madison, WI, USA) and with a Victor 3 Multilabel Counter (PerkinElmer, Wellesley, MA, USA).

### Wnt signaling assays

Recombinant Wnt 1 (Peprotech), Wnt3 (Novus Biologicals, Littleton, CO, USA), and Wnt3a (Peprotech) were used at the final concentration of

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50 ng $mL^{-1}$. Recombinant mouse sFRP-2 and sFRP-3 (R&D systems, Minneapolis, MN, USA) were used at the final concentration of 500 ng $mL^{-1}$. Assays for proliferation and luciferase activity were performed 48–72 h postincubation with Wnt signaling reagents.

### Statistical analysis

Experiments were performed in triplicate. Statistical analysis of mean differences between groups was performed by Student's t-test or two-way ANOVA followed by a Bonferroni *post hoc* analysis (GraphPad Prism, San Diego, CA, USA). Significance was set for *P*-values < 0.05.

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## Author contributions

C.J.M., R.A.A, and B.K.K. designed the research. C.J.M., L.B., Y.J., M.E.H, L.Z., M.R. H.W, and M.R. performed research. C.J.M., L.B., Y.J., R.A.A, and B.K.K. analyzed the data. C.J.M. and B.K.K. wrote the paper.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1** NPCs isolated from 13-month DG morphologically resemble 3-month NPCs and do not show increased levels of cell death or cell heterogeneity.

**Fig. S2** Late passage 13-month NPC have a normal karyotype.

**Fig. S3** Survivin levels directly correlate with NPC proliferation.

**Fig. S4** Survivin levels are higher in NPCs compared with their differentiated progeny.

**Fig. S5** Potent anti-apoptotic genes do not compensate for survivin deficiency in NPCs.

**Fig. S6** Survivin expression is not required for maintenance of neurons and astrocytes.

**Fig. S7** Astrocte-conditioned medium from 13-month astrocytes inhibits Wnt signaling in NPCs.

**Table S1** Comparative quantitative RT-PCR analysis of cell cycle gene levels between 3- and 13-month NPCs.

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