Astroglia induce neurogenesis from adult neural stem cells

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During an investigation of the mechanisms through which the local environment controls the fate specification of adult neural stem cells, we discovered that adult astrocytes from hippocampus are capable of regulating neurogenesis by instructing the stem cells to adopt a neuronal fate. This role in fate specification was unexpected because, during development, neurons are generated before most of the astrocytes. Our findings, together with recent reports that astrocytes regulate synapse formation and synaptic transmission, reinforce the emerging view that astrocytes have an active regulatory role—rather than merely supportive roles traditionally assigned to them—in the mature central nervous system.

New cells are generated throughout the central nervous system (CNS) well into adulthood in all mammals, including humans[1–4]. Adult neurogenesis occurs, however, only in two specific brain regions: the subventricular zone and the hippocampal subgranular zone[1–4]. Outside these two regions, proliferating cells give rise to new glia but not to neurons in the intact adult CNS[2,4–7]. Contrary to what one might anticipate, however, proliferating cells isolated from many regions in the adult brain[8–14], including non-neurogenic regions[11–14], can give rise to neurons both *in vitro*[8–14] and after grafting back to neurogenic regions *in vivo*[15,16]. These findings lead to an emerging view that neural stem cells may be widely distributed in the adult CNS and that the local environmental cues dictate their fate choice. Little is known at present about the mechanisms for the fate specification of adult neural stem cells[3].

In this study, we exploited the advantages of cell culture systems to investigate the contributions made by different cell types to the fate specification of fibroblast growth factor-2 (FGF-2)-dependent stem cells derived from adult hippocampus. We demonstrate that mature astrocytes from postnatal hippocampus promote neurogenesis, whereas neurons increase oligogenesis by the same adult stem cells. Using a quantitative approach, we show that hippocampal astrocytes actively regulate adult neurogenesis both by instructing neuronal fate commitment and by promoting proliferation of adult neural stem cells. Moreover, the effects of astrocytes are regionally specified: astrocytes from adult hippocampus retain the potential to promote neurogenesis, but astrocytes from adult spinal cord do not. This finding of active roles for astroglia in adult neurogenesis is unexpected and raises the possibility that neuronal production in the mature brain is regulated, at least in part, by the regional properties of astrocytes.

Differentiation of adult stem cells

To mark the progeny of stem cells, we infected clonal derived neural stem cells isolated from the hippocampus of adult rat with a retrovirus engineered to express green fluorescent protein (GFP), and then selected GFP-expressing (GFP^+) cells for this study[10,17]. These FGF-2-dependent, GFP^+ adult cells retain their stem cell properties. First, they can give rise to all three principal CNS cell types, as defined by cell-type-specific markers for neurons ($Tuj1^+$, $MAP2ab^+$), oligodendrocytes ($GalC^+$, RIP^+), or astrocytes ($GFAP^+$), both *in vitro*[10,17] (Fig. 1) and *in vivo* after grafting back to adult hippocampus[15]. Second, most of these cells actively proliferate in the presence of 20 ng ml^{-1} FGF-2, as shown by incorporation of bromodeoxyuridine (BrdU) during a 36-h period ($97.9 \pm 0.6\%$, $n = 3$; Fig. 1a). Third, these adult stem cells are positive for nestin ($99.3 \pm 0.2\%$, $n = 3$; Fig. 1a)[18], an immature cell marker, but negative for markers of many differentiated cell types (Fig. 1d).

These adult neural stem cells, unlike some neural stem cells derived from the developing brain[19], appear to require exogenous factors to promote fate commitment to a neuronal lineage under our experimental conditions. This conclusion is based on the observation that the adult stem cells rarely differentiate spontaneously into neurons as defined by expression of MAP2ab, and only infrequently into oligodendrocytes (RIP^+) or astrocytes ($GFAP^+$), when cultured at a low density in a defined medium free of serum and FGF-2 (Fig. 1b, d). Previously, retinoic acid (0.5 μM) and fetal bovine serum (FBS, 0.5%) were shown to induce limited neuronal differentiation of these adult stem cells (Fig. 1d)[17].

To dissect out the contributions made by different cell types to the fate choice of adult-derived stem cells, we cultured FGF-2-dependent GFP^+ adult stem cells on a feeder layer of primary neurons and astrocytes derived from neonatal hippocampus in a defined serum-free medium. We found that within six days after co-culture the GFP^+ adult stem cells gave rise to significant numbers of neurons ($MAP2ab^+$), as well as to some oligodendrocytes (RIP^+), astrocytes ($GFAP^+$) and undifferentiated ($nestin^+$) cells (Fig. 1c). This effect of environment on neurogenesis is robust: there was an approximate eightfold increase in the fraction of $MAP2ab^+$ and GFP^+ neurons in co-culture as compared with that for laminin- or poly-lysine-coated substrates in parallel cultures; however, fractions of $GFAP^+$ and GFP^+ astrocytes or RIP^+ and GFP^+ oligodendrocytes were not significantly changed (Fig. 1d). In contrast, a feeder layer of primary fibroblasts derived from rat skin[20] showed no significant effect on neurogenesis (Fig. 1d). Thus, factors from primary hippocampal neurons and/or astrocytes can specifically promote neurogenesis from these adult neural stem cells in the defined medium free of serum.

Differential effects of astrocytes and neurons

We next addressed the question of which cell type is necessary to promote neurogenesis—neurons, astrocytes or both? To determine whether neurons have a large role in promotion of neurogenesis, we prepared cultures enriched in primary neurons from neonatal hippocampus, in which over 70% of total cells were neurons ($MAP2ab^+$) and less than 20% were astrocytes ($GFAP^+$). When adult stem cells were plated in these neuron-enriched cultures, we did not observe a significant increase in neurogenesis as compared to stem cells plated on laminin-coated substrates (Fig. 2a, c), demonstrating that these primary neurons are not sufficient to

promote neurogenesis from adult stem cells under these conditions. Notably, the fraction of oligodendrocytes (RIP^+ and GFP^+) in neuron-enriched cultures increased significantly (Fig. 2a, c), indicating that primary neurons promote oligodendrocyte production from the adult neural stem cells[21]. When we used primary neurons specifically from neonatal dentate gyrus, instead of neurons from the whole hippocampus, we observed a similar increase in oligodendrocyte production without an increase in neurogenesis (data not shown).

We next examined whether astrocytes alone are sufficient to promote neurogenesis under our experimental conditions. We prepared astrocytes derived from neonatal hippocampus ($GFAP^+$: $95 \pm 3\%$; $n = 3$) without detectable primary oligodendrocytes (RIP^+) or neurons ($MAP2ab^+$) within six days under our co-culture conditions (see Methods). We observed a greater than tenfold increase in the percentage of neurons ($MAP2ab^+$ and GFP^+) produced from adult stem cells on a feeder layer of these astrocytes, as compared to those cultured on laminin-coated substrates in parallel cultures (Fig. 2b, c). Thus, primary hippocampal astrocytes alone are sufficient to promote neurogenesis from these adult stem cells. Astrocytes produce a variety of soluble and membrane-associated factors that influence multiple functions of the CNS[22]. To examine whether the effects of astrocytes on neurogenesis depend on diffusible or membrane-bound factors, we plated adult stem cells on coated substrates conditioned by primary astrocytes without contact[23], or directly on lightly fixed astrocytes[24]. We found significantly more neurons ($MAP2ab^+$ and GFP^+) in both conditions than we observed on coated substrates in parallel cultures (Fig. 2c). These results indicate that both diffusible and membrane-bound factors are responsible for promoting neurogenesis from these adult stem cells.

Survival and proliferation

The effects of hippocampal astrocytes on neurogenesis of adult stem cells could be due to some combination of enhanced neuronal survival, increased proliferation of progenitors, and/or instruction of fate commitment by the adult stem cells to a neuronal lineage. We can determine by direct measurements whether astrocytes promote cell survival (measure the rate of cell death) or promote proliferation of progenitors (measure the rate of cell generation). To determine whether either of these two mechanisms has a role in increased neurogenesis, we established the time course of neurogenesis (defined by the expression of Tuj1, an early neuronal marker). As shown in Fig. 3a, there seems to be a constant production of $Tuj1^+$ and GFP^+ neurons in co-culture with astrocytes, and the effects of astrocytes were observed as early as 1 day after plating. In addition, BrdU labelling experiments showed that some cells were still dividing during the four-day experiments (Fig. 3a).

First, we asked whether a decrease in neural precursor or neuronal death could explain the increased number of neurons ($Tuj1^+$) in co-culture with astrocytes, cells that are known to promote neuronal survival in long-term cultures[25]. To quantify cell death of the progeny of adult stem cells, we used Hoechst 33342 ($2\,\mu g\,ml^{-1}$) to visualize fragmented nuclei characteristics of apoptotic cells in live (Fig. 3b), rather than fixed, cultures; living cultures were used to avoid underestimating cell death owing to detachment of dying and/or dead cells from the culture substrates. This assay revealed a rather small percentage of dying/dead cells and no significant difference between different conditions at any time examined for these short-term cultures (Fig. 3b). Thus, better neuronal survival does not have a significant role in the increased net neurogenesis observed in these short-term co-cultures with astrocytes.

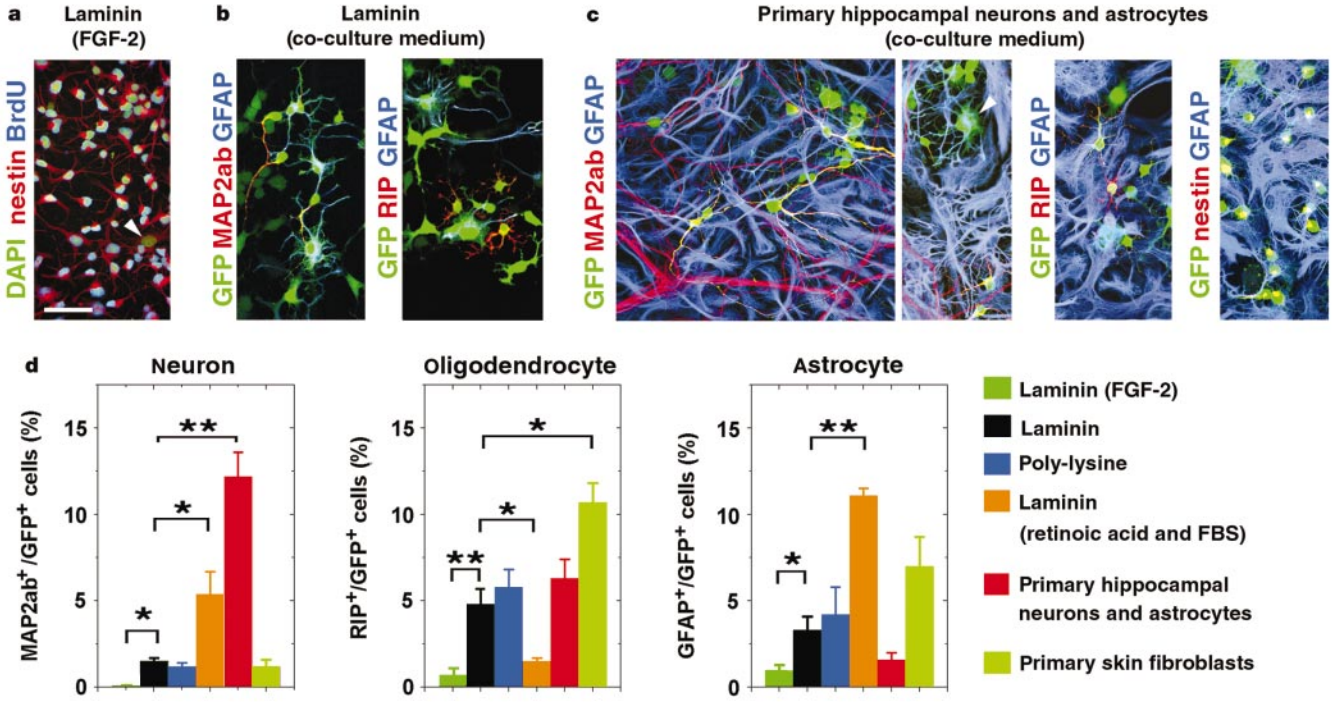

Figure 1 Differentiation of adult neural stem cells in a defined medium without serum. **a**, Proliferation of GFP^+ adult stem cells in the presence of FGF-2. Cells were cultured on laminin-coated substrates in the presence of 2.5 μM BrdU and 20 ng ml^{-1} FGF-2 for 36 h, and stained for BrdU and nestin. An arrowhead marks a $nestin^+$ $BrdU^-$ cell. Scale bar, 50 μm. DAPI, 4,6-diamidino-2-phenylindole. **b**, **c**, Differentiation of GFP^+ adult stem cells cultured on laminin-coated substrates (**b**) or with neonatal hippocampal neurons and astrocytes (**c**). Cells in six-day cultures were stained for markers for neurons (MAP2ab), astrocytes (GFAP), oligodendrocytes (RIP), or immature cells (nestin), respectively. An arrowhead marks a $GFAP^+$ and GFP^+ astrocyte. **d**, Quantification of six-day cultures. Data shown are mean values ± s.e.m. from four to eight experiments in parallel cultures. Significant differences from the control group (laminin) are marked with a single ($P < 0.05$, *t*-test) or double ($P < 0.01$, *t*-test) asterisk.

Next, we examined whether progenitor proliferation might contribute to the observed neurogenesis. Parallel cultures of GFP$^+$ adult stem cells were incubated with BrdU (2.5 μM) for 24 h on days 0, 1, 2 and 3 after plating, immediately followed by fixation and analysis of total cell numbers and BrdU incorporation. For GFP$^+$ stem cells on laminin-coated substrates, we observed a gradual decline in the percentage of cells that were proliferating (GFP$^+$ and BrdU$^+$) during the four-day experiments (Fig. 3c). For those stem cells co-cultured with astrocytes, the fraction of GFP$^+$ cells that divided during the first 24-h period was similar to that found on laminin-coated substrates, but became significantly higher at later time points (Fig. 3d). These observations indicate that factors from astrocytes do promote proliferation of adult neural progenitors. Consistent with results from BrdU labelling, we observed significantly more GFP$^+$ cells in co-culture with astrocytes than on laminin-coated substrates after two days in culture (Fig. 3d). Time-lapse experiments confirmed that there was no significant difference in cell death between different culture conditions within the four-day experiments, and that there was an increase in proliferation for those co-cultured with astrocytes (Fig. 3e). Taken together, these results demonstrate that at least part of the effect of astrocytes on neurogenesis is due to proliferation of neural progenitors.

Neuronal fate commitment

Is the entire astrocytic effect on neurogenesis due to the proliferation of progenitors or do the astrocytes also instruct progenitors to adopt a neuronal fate? To address this question, we developed a mathematical description of neurogenesis (as shown in Fig. 4a) that permits us to separate instructive effects from other possibilities. In our quantitative description of the process, we defined α_j as the proliferation rate (per day) of progenitors for the jth day in culture; β_j as the rate of conversion from progenitors (Tuj1$^-$) to neurons (Tuj1$^+$) during that day; and δ_j as the overall rate of cell death per day. The proliferation rate, α_j, is measured by the percentage of GFP$^+$ cells that incorporate BrdU in a 24-h period (Fig. 3d). This measured rate was used to predict the total GFP$^+$ cell numbers that had been counted in the same cultures. No significant difference was found for total GFP$^+$ cell numbers between direct measurements (Fig. 4b, filled symbols) and theoretical prediction (Fig. 4b, open symbols). The death rate, δ_j, was measured directly by using

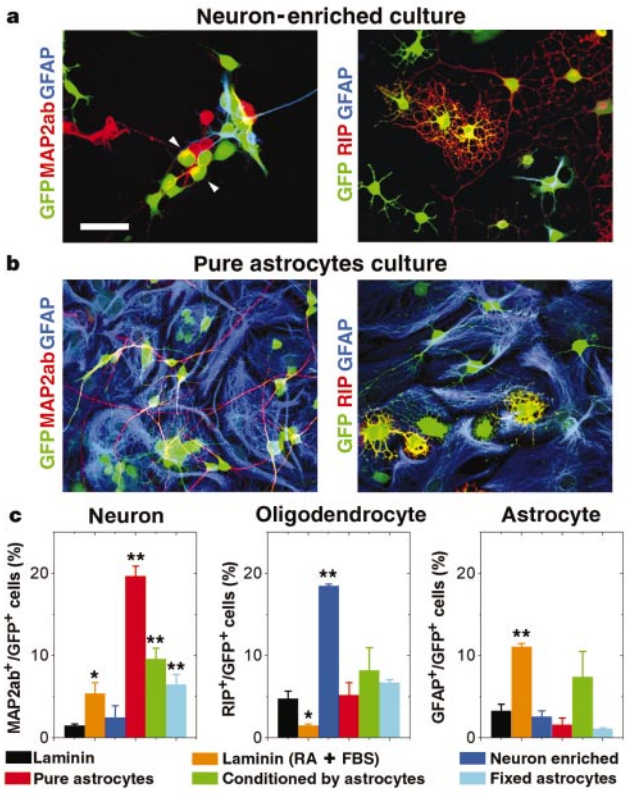

Figure 2 Distinct effects of primary astrocytes and neurons on the fate choice of adult neural stem cells. **a**, **b**, Differentiation of GFP$^+$ adult neural stem cells in hippocampal neuron-enriched (**a**) or pure astrocyte cultures (**b**). Cells in six-day cultures were stained for MAP2ab, GFAP or RIP, respectively. Arrowheads mark GFP$^+$ and MAP2ab$^+$ neurons. Scale bar, 25 μm. **c**, Quantification of six-day cultures. Data shown are mean values ± s.e.m. from four to eight independent experiments in parallel cultures. Significant differences from the control group (laminin) are marked with a single ($P < 0.05$, *t*-test) or double ($P < 0.01$, *t*-test) asterisk. RA, retinoic acid.

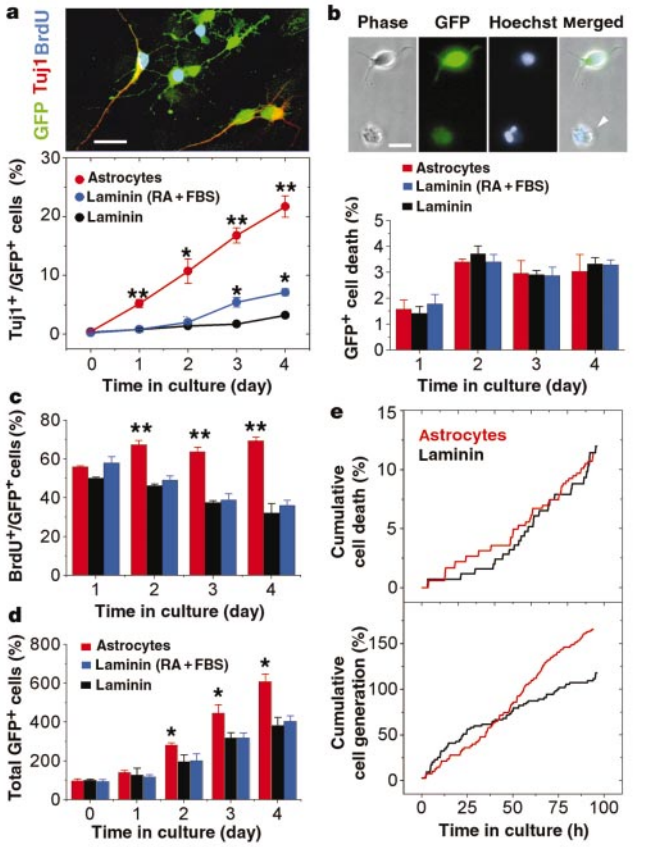

Figure 3 Effects of astrocytes on the survival, proliferation and neuronal fate commitment of adult neural stem cells. **a**, Time course of neurogenesis from adult neural stem cells. Cells cultured on hippocampal astrocyte, or laminin-coated substrates in the absence or presence of retinoic acid (RA, 0.5 μM) and fetal bovine serum (FBS, 0.5%), were fixed at different time points and stained for an early neuronal marker, Tuj1. The top panel shows cells stained for Tuj1 and BrdU in the co-culture (four days) that was pulsed with BrdU (0.5 μM) for 24 h at day 0. **b**, Cell death in different culture conditions as assessed daily by live staining with Hoechst 33342 (2 μg ml^{-1}). The top panel shows example images in phase, GFP fluorescence, Hoechst 33342 fluorescence and merged. An arrowhead marks a dying cell. Scale bar, 2 μm. **c**, Proliferation of GFP$^+$ cells assessed by BrdU incorporation. Parallel cultures were incubated with BrdU (2.5 μM) on different time points for 24 h, immediately followed by fixation and staining for BrdU. All data are mean values ± s.e.m. from four to six independent experiments in parallel cultures. Significant differences from the control group (laminin) are indicated by a single ($P < 0.05$, *t*-test) or double ($P < 0.01$, *t*-test) asterisk. **d**, The number of total GFP$^+$ cells in different culture conditions. All data (same set as in **c**) were normalized to the total cell number on laminin-coated substrates at day 0 (4 h after plating). **e**, Cell generation and cell death of GFP$^+$ cells under different conditions as assessed by time-lapse microscopy over four days. Images were acquired every 15 min. Death and generation of cells were quantified on each set of sequential images.

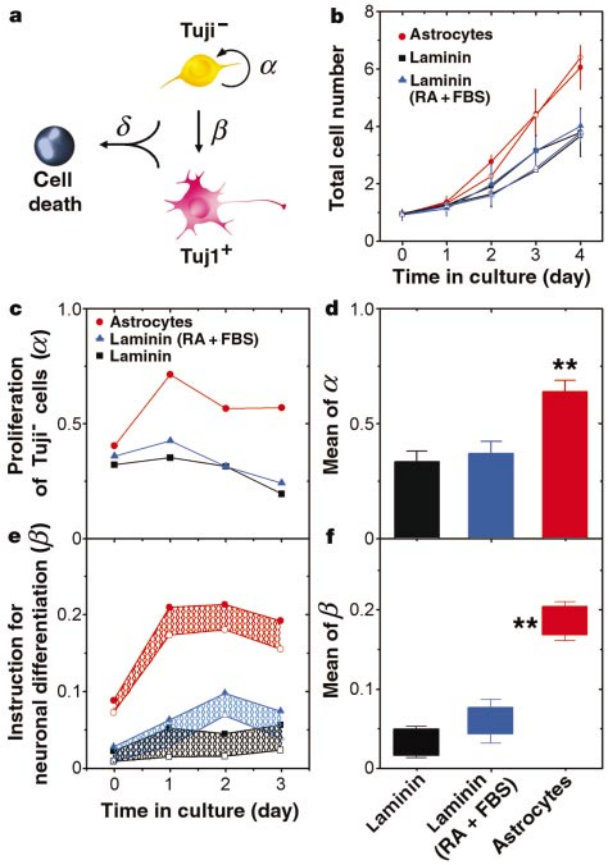

Figure 4 Astrocytes increase the rates of proliferation and neuronal fate commitment of adult neural stem cells. **a**, The states of progeny of adult neural stem cells in culture. α, cell division rate (per day) for non-neurons (Tuj1$^-$); β, conversion rate of progenitors (Tuj1$^-$) to neurons (Tuj1$^+$); δ, cell death rate for all cells. **b**, Comparison of the total cell numbers from direct measurements (filled symbols) and theoretical prediction (open symbols) (see Methods). No significant differences were found between these two conditions in all culture conditions. **c**, **d**, The cell division rates for Tuj1$^-$ cells (α). The time course of α (**c**) and the mean values ± s.e.m. for the last three days (**d**) is shown. **e**, **f**, The conversion rates of Tuj1$^-$ cells to Tuj1$^+$ cells (β). The upper limits (filled symbols) and lower limits (open symbols) for β in each condition are shown (**e**). The mean values ± s.e.m. for the last three days are indicated (**f**). Significant differences are indicated with a double asterisk ($P < 0.01$, *t*-test).

Hoechst 33342 dye in live cultures (Fig. 3b).

We could not, however, determine what fractions of the dying and dead cells were neurons. Counting the number of GFP$^+$ neurons (Tuj1$^+$) each day did permit us to estimate upper and lower limits on the conversion rate (β_j) of progenitors to neurons (Fig. 4e). These estimates were made with the limiting assumptions that either no neurons died or all cells that died were neurons. On the basis of these estimates, we conclude that adult stem cells proliferate about twice as rapidly on astrocytes (Fig. 4d) as those on laminin-coated substrate with or without the addition of retinoic acid (0.5 μM) and FBS (0.5%). Furthermore, and most importantly, the rate of conversion of progenitors to neurons is about six times higher for adult stem cells co-cultured with astrocytes (Fig. 4f). Thus, we are able to demonstrate quantitatively that hippocampal astrocytes increase the rates of both proliferation and neuronal fate commitment of FGF-2-dependent adult neural stem cells.

Developmental and regional specificity

These findings raised the question of whether distinct subtypes of glial cells are located in specific regions to regulate neurogenesis in the adult brain. To examine the effects of local environment on adult neurogenesis *in vivo*, we labelled proliferating cell populations, including the adult neural stem cells, in the dentate gyrus of adult hippocampus by pulsed injection of BrdU. Most BrdU-labelled cells were located near the inner molecular layer in intimate association with GFAP$^+$ astrocytes in the dentate gyrus of adult hippocampus, and were negative for any neuronal and glial markers at this stage (Fig. 5). Within a week of cell division, some progeny of proliferating cells began to express markers common for immature neurons and glia[20]. Notably, as newly generated granule neurons mature, they migrate into the granule layer[26], where the cell bodies of granule neurons are densely packed with less exposure to GFAP$^+$ astrocytes. Recently, a vascular niche for adult hippocampal neurogenesis was identified[20]. Astrocytes are well known to be in close association with vasculature[27,28], so they may provide an important part of the cellular elements that regulate adult neurogenesis in the dentate gyrus.

To examine whether adult astrocytes also regulate neurogenesis, we co-cultured GFP$^+$ adult stem cells with astrocytes derived from hippocampus of adult rats (Fig. 6a). We found that significantly more GFP$^+$ and MAP2ab$^+$ neurons are present in co-culture with adult hippocampal astrocytes as compared with those on coated substrates or skin fibroblasts (Fig. 6c); however, the promotion of neurogenesis by astrocytes derived from adult hippocampus is about half as effective as astrocytes derived from neonatal hippocampus (Fig. 6c). Thus adult hippocampal astrocytes retain the

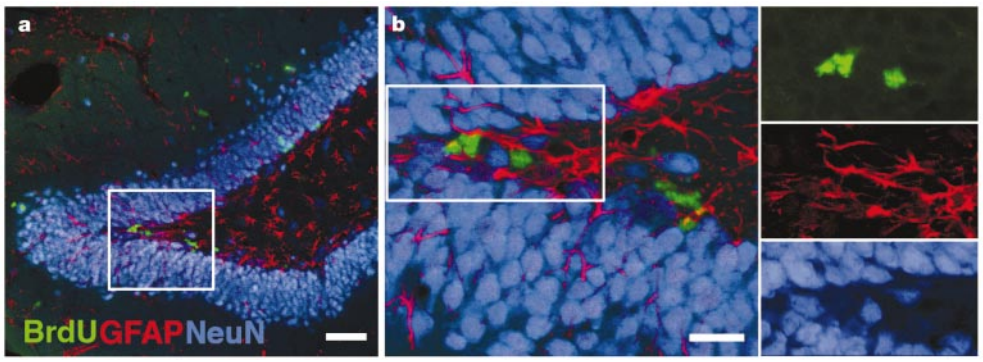

Figure 5 Intimate association of proliferating cells with GFAP$^+$ astrocytes in the dentate gyrus of adult hippocampus. Three-month-old rats were pulsed with BrdU (50 mg kg^{-1}, three times per day for three days). Tissues were fixed one day after the last BrdU injection and stained for BrdU (green), astrocyte marker GFAP (red) and neuronal marker NeuN (blue). The region in the white box in **a** is shown at a higher magnification in **b**. Scale bar, 10 μm (**a**); 2 μm (**b**).

ability to promote neurogenesis from adult stem cells, but are less effective than the neonatal astrocytes, an effect consistent with the *in vivo* observation that the rate of neurogenesis decreases with age[29].

As noted above, active adult neurogenesis occurs in the subventricular zone and the subgranular zone of the dentate gyrus in hippocampus, but not in other regions of the intact CNS[2,4–7]. Multipotent stem cells can, however, be isolated from many non-neurogenic regions[11–14], such as adult spinal cord[11,12]. Our observation that hippocampal astrocytes can promote neurogenesis by adult stem cells raises the possibility that neurogenesis is absent in the adult spinal cord because the appropriate astrocytic signals are not present there. To examine this possibility, we co-cultured GFP^+ adult stem cells with astrocytes from neonatal or adult spinal cord (Fig. 6b) and found that neonatal astrocytes from spinal cord produced some neuronal differentiation of these adult stem cells, but that adult astrocytes from spinal cord do support neurogenesis (Fig. 6c). In contrast, there is an increase in the fraction of $GFAP^+$ and GFP^+ astrocytes and RIP^+ and GFP^+ oligodendrocytes in co-culture with spinal cord astrocytes (data not shown). These results demonstrate that astrocytes from different CNS regions at several developmental stages exhibit different capabilities for regulating the fate choice of adult stem cells.

Discussion

In this study, we have examined the regulation of fate choices of FGF-2-dependent, adult-derived stem cells by different cell types that may exist in the niche of proliferative cells in adult brains. We demonstrated that primary neurons promote oligodendrocyte differentiation whereas astrocytes from postnatal hippocampus can actively regulate neurogenesis from adult neural stem cells. Using a quantitative approach, we established that factors from astrocytes increase the rates of neuronal fate commitment and proliferation of progenitors by approximately sixfold and twofold, respectively (Fig. 4).

During development of the mammalian CNS, neurons and glial cells arise from multipotent progenitors in a stereotyped sequence through which neurons are generated first, primarily during the embryonic period, followed by glia, most of which differentiate after most neurons are generated[3,27,28]. Astrocytes are known to support the proliferation, survival and maturation of developing neurons and neuroblasts that have already committed to neuronal lineages[27,28,30], as well as to stimulate neurogenesis from subventricular zone progenitors[24]. In the mature mammalian brain, astrocytes constitute nearly half of the total cells, providing structural, metabolic and tropic support for neurons[27,28]. Active, rather than supportive, roles for astrocytes in the adult CNS have been proposed only recently. For example, astrocytes have been reported to induce and/or stabilize CNS synapses[31,32] and may be capable of integrating neuronal inputs and modulating synaptic activity[33–36]. In contrast to neurogenesis during development—when most astrocytes are not yet generated—adult neural stem cells reside in a significantly different environment. In the dentate gyrus of adult hippocampus, astrocytes are in intimate contact with proliferating cells *in vivo* (Fig. 5). Our results suggest active, rather than supportive, functions for astrocytes in adult brains: hippocampal astrocytes instruct the neuronal fate commitment of adult stem cells. Radial glia, which are a subpopulation of precursors generated before embryonic neurogenesis, were recently shown to give rise to neurons[37,38]. It remains to be examined whether radial glia also directly regulate neurogenesis during development.

Active neurogenesis is limited to discrete regions of intact CNS of adult mammals[1–4]. Local environments seem to dictate the fate choice of adult stem cells[6,15,16,39]. For example, neural stem cells derived from adult hippocampus or spinal cord gave rise to neurons after grafting to the dentate gyrus, but not in the spinal cord[16]. In regions of adult mammalian neocortex that do not normally undergo any neurogenesis, endogenous neural precursors can differentiate *in situ* into neurons after induced synchronous apoptotic degeneration of corticothalamic neurons in the surroundings[6]. Consistent with *in vivo* observations[5], we found that astrocytes from adult spinal cord, one of the non-neurogenic regions, are ineffective in promoting neurogenesis from adult stem cells. These results suggest that hippocampal astrocytes provide a unique niche for adult neurogenesis and present an intriguing possibility that the capability for adult neurogenesis might, in part, be due to certain signals provided by regionally specified astrocytes in the adult CNS. □

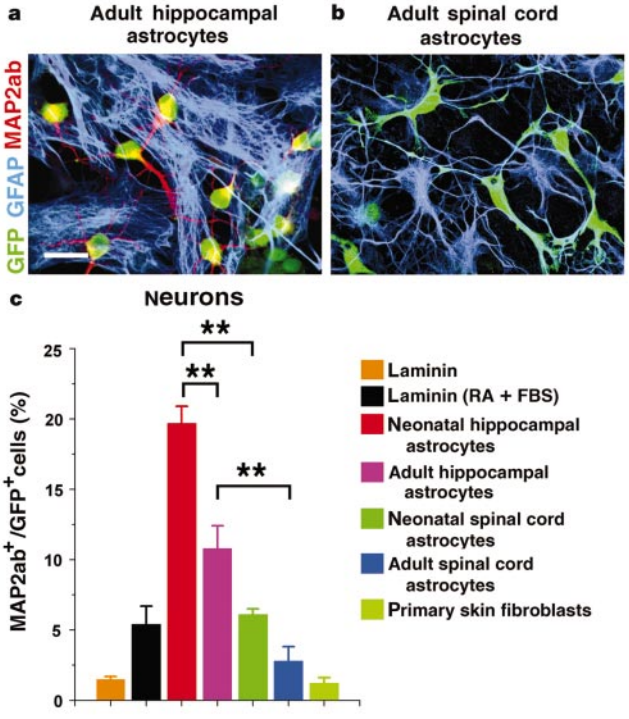

Figure 6 Mature astrocytes from adult hippocampus, but not adult spinal cord, promote neurogenesis from adult stem cells. **a**, **b**, Differentiation of GFP^+ adult neural stem cells in co-culture with astrocytes derived from adult hippocampus (**a**) or adult spinal cord (**b**). Cells in six-day cultures were stained for MAP2ab and GFAP. Scale bar, 5 μm. **c**, Quantification of the percentage of $MAP2ab^+$ and GFP^+ neurons in different conditions (six-day culture). Data shown are mean values ± s.e.m. from four to eight experiments in parallel cultures. Significant differences between results for astrocytes from hippocampus and spinal cord are indicated by a double asterisk ($P < 0.01$, *t*-test).

Methods

Cell cultures

The clonal derived stem cells isolated from hippocampus of adult Fisher 344 rats used in this study have been characterized previously[10,17]. They were infected with retrovirus to express GFP, selected and propagated on laminin-coated flasks in DEME/F12 medium containing N2 supplement, L-glutamine (2 mM) and FGF-2 (20 ng ml^{-1}) as previously described[10,17]. For differentiation, the stem cells were trypsinized and plated in a serum-free defined medium (co-culture medium) containing MEM, N2 supplement, sodium pyruvate (1 mM), glucose (0.2 M) and L-glutamine (2 mM).

Primary astrocytes from hippocampus or spinal cord of postnatal day 0 (P0) or adult (older than six weeks) rats were prepared essentially as described[23,40–42]. Importantly, confluent astrocytes were treated with cytosine arabinoside (20 μM) for 72 h to eliminate proliferating cells, followed by recovery in fresh medium for 24 h. These steps may be repeated to further eliminate other cell types[41,42]. Under these conditions, most of the cells were $GFAP^+$ and/or S-100β$^+$ astrocytes with little contamination by RIP^+ or $MAP2ab^+$ cells within 6 days under the co-culture conditions. We prepared primary neurons from P0 hippocampus as described previously[40]. Primary neurons were plated at 10,000 cells cm^{-2} on a confluent feeder layer of astrocytes from P0 hippocampus in the co-culture medium. For neuron-enriched cultures, P0 neurons from whole hippocampus, or only from dentate gyrus, were plated at 100,000 cells cm^{-2} on poly-lysine-coated coverslips and cultured for two days in MEM containing N2 supplement, horse serum (10%), sodium pyruvate (1 mM), glucose (0.2 M) and cytosine arabinoside (5 μM). We then changed the medium

to fresh co-culture medium. These cultures survived for more than two weeks with over 70% of total cells as MAP2ab$^+$ neurons and less than 20% as GFAP$^+$ astrocytes. Primary skin fibroblasts from Fischer 344 rats[20] were cultured in DMEM containing 10% FBS and did not contain any detectable MAP2ab$^+$, GFAP$^+$ or RIP$^+$ cells.

For co-culture experiments, substrate cells were cultured on poly-lysine- and collagen-coated coverslips until they were confluent. Fixed astrocyte feeder layers were prepared by light fixation with cold 70% ethanol at −20 °C for 30 min[24]. For co-cultures using Banker culture, astrocytes were cultured on the bottom of 24-well plates, while laminin-coated coverslips were placed in the same well without contact[23]. Substrate cells or coated coverslips were incubated in co-culture medium overnight before GFP$^+$ stem cells were plated at 10,000 cells cm^{-2} in parallel for all culture conditions. Cultures were fed every other day. We added BrdU (2.5 μM) in some cultures to label dividing cells.

Immunocytochemistry and quantification

Cultures were processed for immunocytochemistry as described previously[10,17]. We used the following primary antibodies: BrdU (1:400, rat; Accurate), nestin (1:1,000, mouse; Pharmingen), α-type III β-tublin (Tuj1, 1:1,000, mouse; BAVCO), MAP2ab (1:250, mouse; Sigma), RIP (1:25, mouse; Hybrodoma Bank), S-100β (1:500, rabbit; Sigma) and GFAP (1:500, guinea-pig; Advance Immuno or 1:1,000, rabbit; Dako). The detection of BrdU in cultured cells required treatment in 1M HCN at 37 °C for 30 min[10]. Images were taken on a Bio-Rad confocal system at an original magnification of ×40 or ×63 with individual filter sets for each channel, and were assembled in PhotoShop.

Fluorescently labelled cells were visualized using confocal microscopy. Positive cells were quantified in at least 20 fields systematically across the coverslips from four to eight independent experiments of parallel cultures. Statistical analysis was performed with Prizm.

Time-lapse recordings

GFP$^+$ stem cells were plated onto different substrates and cultured for four days in recording chambers with 5% CO_2 at 37 °C on a microscope stage. Both phase and fluorescent images were captured every 15 min. Cell death and cell generation can be clearly identified on sequential images and thus analysed for a period of four days.

Quantitative analysis of neurogenesis from adult stem cells

The state diagram describing our experiments is shown in Fig. 4a. N_{j+1} is the total number of cells present at the start of the $(j+1)$ day in culture, and n_{j+1} is the total number of neurons (Tuji$^+$ cells) present at that time. α_j is the cell division rate for non-neurons (Tuji$^-$ cells) on the jth day; β_j is the conversion rate of progenitors to neurons for that day; and δ_j is the death rate of all cells. For example, the number of cells that die during the jth day in culture is $\delta_i N_j$—the other rates are defined in an analogous way. The total number of cells present at the start of the $(j+1)$ day, then, is given by the equation $N_{j+1} = (1+\alpha_j)\times(N_j - n_j) + n_j - \delta_j N_j$. The first term on the right gives the contribution to the number due to progenitor division, the second term is the number of neurons present (assumed not to divide), and the third term specifies the contribution due to cell death. The cell division rate, α_j, is also given by the equation $N^*_{j+1} = 2\alpha_j(N_j - n_j)$, where N^*_{j+1} is the number of BrdU-labelled cells at the start of the $(j+1)$ day. Finally, the number of neurons present at the start of the $(j+1)$ day is given by the equation $n_{j+1} = n_j + \beta_j(N_j - n_j) - \delta^n_j n_j$, where δ^n_j is the death rate of neurons and the quantity $\delta^n_j n_j$ gives the number of neurons that die during the jth day. This equation states that the number of neurons present at the start of the $(j=1)$ day is made up of the ones present at the start of the jth day (n_j), plus the number of neurons produced by conversion of progenitors, $\beta_j(N_j - n_j)$, minus the number of neurons that died. We do not know this death number, but the inequality $\delta_j N_j \geq \delta^n_j n_j \geq 0$ places limits on the neuronal death. These equations, together with the measured numbers of total GFP$^+$ cells, neurons, BrdU-labelled cell numbers, and cell death each day permit us to obtain α_j, β_j and δ_j (see Fig. 4).

In vivo BrdU labelling

Three-month-old Fischer 344 rats were injected intraperitoneally nine times with BrdU (50 mg kg^{-1}) over the course of three days, and tissues were fixed one day after the last injection. Tissues were processed for immunostaining as described previously[20].

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Competing interests statement

The authors declare that they have no competing financial interests.

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