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Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling

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Abstract

Neurogenesis in the adult hippocampus involves activation of quiescent neural stem cells (NSCs) to yield transiently amplifying NSCs and progenitors, and ultimately neurons that affect learning and memory. This process is tightly controlled by microenvironmental cues, though few endogenous factors are known to regulate neuronal differentiation. While astrocytes have been implicated, their role in juxtacrine (i.e. cell-cell contact-dependent) signaling within NSC niches has not been investigated. We show that ephrin-B2 presented from rodent hippocampal astrocytes regulates neurogenesis *in vivo*. Furthermore, clonal analysis in NSC fate-mapping studies reveals a novel role for ephrin-B2 in instructing neuronal differentiation. Additionally, ephrin-B2 signaling, transduced by EphB4 receptors on NSCs, activates β-catenin *in vitro* and *in vivo* independent of Wnt signaling and upregulates proneural transcription factors. Ephrin-B2^{+} astrocytes thus promote neuronal differentiation of adult NSCs through juxtacrine signaling, findings that advance our understanding of adult neurogenesis and may have future regenerative medicine implications.

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Author Contributions

C.P. designed and executed initial *in vitro* ephrin ligand studies. R.A. performed *in vitro* immunocytochemistry, and A.C. performed *in vivo* immunohistochemistry. R.A. designed all ephrin-B2 *in vivo* gain- and loss-of-function experiments, and A.C. and J.B. equally assisted in conducting the experiments. RNAi vectors were designed by R.A. and K.L., cloned by K.L. and P.S., and R.A. validated vectors experimentally and conducted *in vitro* RNAi/co-culture experiments. A.C. validated expression of shRNA vectors in hippocampal astrocytes. RA conducted mouse breeding, and RA designed and conducted fate mapping experiments with significant input and assistance from AC. AC conducted clonal analysis experiments, designed and conducted all experiments related to *in vivo* β-catenin activity studies, and analyzed tissue sample from both fate mapping and β-catenin activity studies. A.C. and C.P., and to a lesser extent R.A., executed the eprhin-B2/GSK3 βS9A/ β-catenin/*Mash1*/*NeuroD1* mechanistic studies. R.A. and A.C. performed statistical analysis. A.C. illustrated Supplementary Fig 8. R.A. and D.S. co-wrote the manuscript, with significant input from A.C. D.S. supervised all aspects of this work, and M.B. provided important scientific input and feedback.

Supplementary information

Supplementary information includes nine figures and one table.

In the mammalian brain, neurogenesis persists throughout adulthood in the subgranular zone (SGZ) of the hippocampal dentate gyrus[1] and the subventricular zone (SVZ) of the lateral ventricles[2]. Within these niches, neural stem cell (NSC) maintenance, proliferation, and differentiation are orchestrated by a delicate balance of microenvironmental cues. The importance of such instructive signals in the niche is supported by the absence of significant neurogenesis in the adult mammalian central nervous system (CNS) outside of these regions[3,4] and by the development of aberrant neurogenesis in patients with pathologies that disrupt the natural chemistry of the brain, for example epilepsy[5], inflammation[6], and neurodegenerative diseases[7].

In the SGZ, adult neurogenesis entails the activation of quiescent Type 1 NSCs to produce mitotic, multipotent Type 2a NSCs[8,9]; the differentiation of the Type 2a NSCs to lineage-committed, proliferative Type 2b neuronal precursors and subsequently Type 3 neuroblasts; and the survival, migration, and maturation of neuroblasts as they differentiate into granule cells that synaptically integrate into the existing neural network[8–10]. Each of these stages is regulated by signals within the niche. For example, bone morphogenic protein[11] and Notch[12,13] signaling modulate the balance between quiescent and proliferative NSCs, and Sonic hedgehog[14], fibroblast growth factor-2[15], vascular endothelial growth factor[16], and Wnt7a[17] regulate NSC proliferation.

While there is thus increasing knowledge of niche factors that regulate NSC division, significantly less is known about key signals that instruct cells in the SGZ to undergo neuronal differentiation[18,19]. Gamma aminobutyric acid (GABA) inputs from local neuronal circuitry[20] and systemic retinoic acid levels[21] modulate NSC neuronal fate commitment. Furthermore, while neuronal differentiation precedes gliogenesis during CNS development, adult hippocampal astrocytes directly induce neuronal differentiation of NSCs *in vitro* via both secreted and membrane-associated factors[22], and the former have since been found to include Wnt3a[23] and potentially additional secreted signals[19]. However, the membrane-bound astrocytic components[22] that may play critical roles in neuronal fate commitment *in vivo* remain unidentified.

Ephrins are a diverse class of glycophosphatidylinositol-linked (ephrinA1–6) and transmembrane (ephrinB1–3) cell surface ligands that bind Eph receptors (EphA1–10 and EphB1–6) on opposing cell membranes to initiate bidirectional signaling[24]. Ephrin/Eph signaling is traditionally known to control the spatial organization of cells and their projections by modulating intercellular attractive and repulsive forces[25]. For example, ephrin/Eph signaling instructs topographical mapping of hippocampo-septal and entorhino-hippocampal projections during development[26] and regulates neuronal dendrite spine morphogenesis and synaptogenesis of adult hippocampal neurons[27].

Recent studies have also indicated that ephrin/Eph signaling plays an earlier role in regulating stem cell behavior. For example, ephrin-A/EphA signaling promotes embryonic telencephalic NSC differentiation[28], and ephrin-B3/EphB3 signaling may exert an anti-proliferative effect on NSCs in the developing SVZ[29]. Within the adult CNS, infusion of ephrin-B2 or EphB2 ectodomains into the lateral ventricles induced SVZ NSC proliferation and disrupted neuroblast migration through the rostral migratory stream[30]. Also, signaling

between ephrin-A2$^+$ neural stem cells, and EphA7$^+$ ependymal cells and putative stem cells, was shown to suppress proliferation of NSCs in the adult SVZ[31]. Within the adult SGZ, ephrin-B3$^+$ neurons in the granule cell layer have been proposed to regulate EphB1$^+$ NSC polarity, SGZ positioning, and proliferation[32]. Similarly, in *Efna5*$^{-/-}$ mice, a decrease in both the proliferation of NSCs and the survival/maturation of newborn neurons in the adult SGZ was observed[33]. Thus, ephrin/Eph signaling has been shown to affect the proliferation, migration, and survival of adult NSCs; however, its potential regulation of NSC fate commitment remains unknown.

Here, we demonstrate that ephrin-B2 presented by hippocampal astrocytes instructs neuronal differentiation of NSCs in the SGZ of the adult hippocampus. Furthermore, ephrin-B2/EphB4 forward signaling induces neuronal differentiation of NSCs by activating β-catenin, independent of Wnt signaling, and inducing transcription of proneural transcription factors. Thus, these findings describe a novel juxtacrine signaling mechanism by which astrocytes actively regulate neuronal differentiation of NSCs during adult neurogenesis.

Results

Ephrin-B2 and EphB4 expression in the SGZ

In the SGZ, Type 1 NSCs express the SRY-box 2 (Sox2) transcription factor and stain for glial fibrilary acid protein (GFAP) and Nestin along radial granule cell layer (GCL)-spanning or horizontal processes[13]. Type 2a NSCs[9] remain Sox2$^+$ and Nestin$^+$ but downregulate GFAP expression, and fate-restricted, proliferative Type 2b neuronal precursors co-express Sox2 and doublecortin (DCX) while downregulating Nestin expression. The latter then mature into (Sox2$^-$/DCX$^+$) Type 3 neuroblasts, which migrate into the GCL to become neuronal specific nuclear protein (NeuN)-positive/DCX$^-$ granule neurons[8,10,11,13]. Alternatively, Type 1 NSCs can differentiate into stellate, Sox2$^-$/GFAP$^+$, hippocampal astrocytes primarily located in the hilus adjacent to the SGZ[10].

As part of a candidate screen, we investigated ephrin-B2 expression within the hippocampal dentate gyrus. Ephrin-B2 antibodies consistently labeled GFAP$^+$ astrocytes in the hilus, the SGZ, and the molecular layer (Fig. 1a,b). In contrast, EphB4, an ephrin-B2 receptor, was expressed by Sox2$^+$/DCX$^-$, Sox2$^+$/DCX$^+$, Sox2$^-$/DCX$^+$, and Sox2$^+$/GFAP$^-$ cells in the SGZ, as well as on the majority of cells in the GCL (Fig. 1a,c,d). Therefore, EphB4 appears to be expressed in Type 2a (Sox2$^+$/DCX$^-$/GFAP$^-$) NSCs and persists as these cells become Type 2b neuronal precursors (Sox2$^+$/DCX$^+$), Type 3 neuroblasts (Sox2$^-$/DCX$^+$), and eventually granule neurons. Some Type 1 NSCs stained EphB4$^+$, though in many EphB4 was not expressed at levels detectable by immunostaining (data not shown). Overall, staining results indicate that astrocytes are a source of ephrin-B2 ligand in the dentate gyrus and suggest that EphB4-expressing NSCs, neuronal precursors, and neuroblasts contact these ligand-expressing astrocytes (Fig. 1e,f).

Ephrin-B2 increases neurogenesis *in vitro* and *in vivo*

Ephrin/Eph signaling begins with clustering of multiple ligand-receptor complexes at sites of cell-cell contact[24]. Thus to initially explore a possible role for ephrin-B2 in regulating

NSC fate, antibody-clustered ephrin-B2/Fc fusion molecules (Fc-ephrin-B2)[34] were added to adult hippocampus-derived NSCs in culture and found to induce a strong, dose-dependent increase in NSC neuronal differentiation (Fig. 2a,b). No biological activity was observed with monomeric, unclustered ephrin-B2/Fc, consistent with previous findings[30,34], and no proliferative effect was observed under any conditions (data not shown). To investigate which receptor mediated Fc-ephrin-B2's activity, the NSCs were pre-incubated with an antibody against EphB2 or EphB4, two known ephrin-B2 receptors, before addition of Fc-ephrin-B2. Inhibition of EphB4, but not EphB2, significantly reduced Fc-ephrin-B2 induction of neuronal differentiation (Fig. 2c), indicating that EphB4 receptors mediate the proneuronal effect of Fc-ephrin-B2. RT-PCR and immunocytochemistry (ICC) results confirmed that hippocampus-derived NSCs express EphB4 receptors (Fig. 2d), in contrast to SVZ NSCs which reportedly do not express EphB4[30].

Given the previous staining and *in vitro* results, we hypothesized that astrocytic ephrin-B2 actively promotes neuronal differentiation of NSCs in the SGZ through juxtacrine signaling, and therefore, we proceeded to investigate Fc-ephrin-B2 activity *in vivo*. Mitotic cells in the brain of adult rats were first labeled with BrdU, followed by bilateral hippocampal injections of PBS, non-clustered ephrin-B2, Anti-Fc antibody without ephrin-B2, or Fc-ephrin-B2 (Fig. 3a). Five days after injection, histology showed an increase in the number of $BrdU^+$ cells in the SGZ of animals injected with Fc-ephrin-B2 compared to animals treated with vehicle or clustering antibody controls (Fig. 3c), mirroring one report in the SVZ[30]. In addition, no difference in the percentage of $BrdU^+$ cells that co-stained as non-radial $GFAP^+$ astrocytes was observed between experimental groups at Day 5 (Supplementary Fig. 1a,b). However, there was a considerable increase in the proportion of $BrdU^+$ cells that co-stained for DCX in animals injected with Fc-ephrin-B2 (80.6% ± 0.87%) as compared to PBS (40.65% ± 4.15%), ephrin-B2 (50.3% ± 3.45%), and Anti-Fc (50.37% ± 9.28%) controls (Fig. 3b,d). These results indicate that ephrin-B2 signaling *in vivo* may regulate early stages of adult hippocampal neurogenesis by modulating NSC proliferation and/or differentiation, but further analysis is required to distinguish between these two possibilities.

Astroctyic ephrin-B2 regulates neurogenesis *in vitro*

Exogenous ephrin-B2 elevates hippocampal neurogenesis, and astrocytes expressing ephrin-B2 contact NSCs in the hippocampus; however, it remains unclear whether astrocytic ephrin-B2 regulates NSC fate. Previous studies have shown that hippocampal astrocytes promote neuronal differentiation of co-cultured NSCs through secretion of soluble Wnt3a and by unidentified membrane-bound signaling molecules[22,23].To determine whether ephrin-B2 is a key component of this membrane-associated activity, we first verified its expression in cultured hippocampal astrocytes. Consistent with the *in vivo* results (Fig. 1), QPCR revealed that hippocampus-derived astrocytes express ephrin-B2 mRNA at levels three orders of magnitude higher than cultured NSCs (Supplementary Fig. 2a). Notably, cultured NSCs down-regulate EphB4 and up-regulate ephrin-B2 expression upon astrocytic differentiation, yet maintain EphB4 expression on their soma upon neuronal differentiation into DCX^+ immature neurons (Supplementary Fig. 2b).

We then analyzed whether RNAi-mediated knockdown of ephrin-B2 in hippocampal astrocytes could compromise their ability to induce neuronal differentiation of NSCs in co-culture. We screened five candidate anti-*efnb2* shRNA sequences expressed under a human and/or a mouse U6 promoter upstream of a human ubiquitin-C promoter eGFP expression cassette (Supplementary Fig. 3). Upon lentiviral delivery, QPCR analysis indicated that two shRNA constructs (*efnb2* shRNA #1 and #2) could knockdown ephrin-B2 mRNA levels in astrocytes by approximately 90 and 85%, respectively (Fig. 4a). Next, BrdU-labeled NSCs (<95%) were seeded on near-confluent layers of mitotically inactivate astrocytes, that were naïve or expressing *efnb2* shRNA #1, #2, or *LacZ* shRNA, and the co-cultures were immunostained after six days. NSC proliferation was insignificant in all experimental groups. However, the percentage of βIII-Tubulin$^+$/BrdU$^+$ cells increased from 6.79 ± 0.62% in NSC-only control cultures to 14.2 ± 0.55% in NSC/ naïve astrocyte co-cultures (Fig. 4b), levels consistent with a prior NSC-astrocyte co-culture study[22], and the neuronally differentiated NSCs were primarily located in close proximity to astrocytes (Fig. 4c). A similar proneuronal effect was maintained by astrocytes expressing the *LacZ* shRNA (15.4 ± 1.12%); however, knockdown of ephrin-B2 expression within *efnb2* shRNA #1 and #2 astrocytes decreased their neuronal instructive potential by ~70%, i.e. to 9.09 ± 0.39% and 9.51 ± 0.11% βIII-Tubulin$^+$/BrdU$^+$ cells, respectively. Intriguingly, this decrease in NSC neuronal differentiation due to ephrin-B2 knockdown is comparable to the previously observed ~55% loss when NSCs were cultured in astrocyte-conditioned medium rather than in co-culture[22]. Furthermore, antibody blockage of EphB4 receptors in the co-culture assay also significantly inhibited the proneuronal effect of naïve astrocytes, resulting in only 10.75 ± 0.87% of NSCs differentiating into βIII-Tubulin$^+$/BrdU$^+$ neurons. Ephrin-B2 is thus a key membrane-presented factor that hippocampal astrocytes employ to instruct neuronal differentiation of NSCs *in vitro*, and EphB4 receptors on NSCs transduce this signal.

Loss of astrocytic ephrin-B2 decreases neurogenesis in SGZ

To determine whether endogenous ephrin-B2 signaling promotes neuronal differentiation *in vivo*, adult rats were injected intrahippocampally with lentiviral vectors encoding *efnb2* shRNA #1, #2, or control *LacZ* shRNA. After two weeks, mitotic cells in the SGZ were labeled with BrdU, and tissue samples were collected after five additional days (Fig. 5a). Within GFP$^+$ regions of the hippocampus, ephrin-B2 levels from rats treated with the *efnb2* shRNA constructs were dramatically lower than in sections from PBS or *LacZ* shRNA treated rats, which exhibited ephrin-B2 expression in patterns similar to GFAP staining (Fig. 5b). We noted that neurons rather than astrocytes expressed GFP in the lentiviral vector-infected hippocampi; however, it was later confirmed through administration of an otherwise identical lentiviral vector in which the human ubiquitin-C promoter was replaced with a GFAP promoter[22] that the low GFP expression initially observed in astrocytes was due to the ubiquitin promoter (Supplementary Fig. 4a,b). In either case, the U6 promoter driving the shRNA expression mediated strong ephrin-B2 knockdown in hippocampal astrocytes (Fig. 5b and Supplementary Fig. 4c).

Next, BrdU$^+$ and DCX$^+$/BrdU$^+$ cells in the SGZ were quantified within GFP$^+$ hippocampi (Fig. 5c). In contrast to results obtained after administration of exogenous ephrin-B2, no significant difference in the number of BrdU$^+$ cells/mm^3 was observed in *efnb2* shRNA #1

(33.2 ± 11.3%) and #2 (22.5 ± 2.50%) vs. *LacZ* shRNA control animals (29.0 ± 7.20%, Fig. 5d), importantly indicating that endogenous ephrin-B2 signaling does not by itself regulate the proliferation of NSCs and Type 2b neuronal precursors. However, consistent with the proneuronal effect of Fc-ephrin-B2 *in vitro* and *in vivo*, the percentage of $BrdU^+$ cells that co-stained for DCX was significantly lower in animals treated with *efnb2* shRNA #1 (32.3 ±4.95%) and #2 (41.6 ± 3.41%) compared to *LacZ* shRNA (59.4 ± 2.33%, Fig. 5e). Therefore, knockdown of ephrin-B2 expression in hippocampal astrocytes decreased neurogenesis in the adult SGZ.

Ephrin-B2 signaling instructs NSC neuronal differentiation

To gain insight into whether neural stem and/or progenitor cell populations are modulated by ephrin-B2, we developed an inducible, conditional, reporter mouse strain to map the fate of $Nestin^+$ NSCs in response to exogenous ephrin-B2. *Nestin-CreER^{T2}* [35] and *R26-stopfl/fl-lacZ* [36] mouse strains were bred to generate mice in which tamoxifen administration induces *lacZ* expression in $Nestin^+$ cells via a transient recombination event. Using adult *Nestin-CreER^{T2}; R26-stop$^{fl/fl}$-lacZ* mice, cells were co-labeled by BrdU and tamoxifen injections prior to an intrahippocampal injection of Fc-ephrin-B2 or the Anti-Fc antibody control, and tissue sections were collected at day 0 from sham mice and at day 5 and 14 post-treatment (Fig. 6a). Quiescent and mitotic cells in the SGZ were labeled $\beta\text{-Gal}^+$ and $BrdU^+$ in a near clonal fashion on day 0 (43.6 ± 7.95% of $\beta\text{-Gal}^+$ cells were also $BrdU^+$; 3.89 ± 1.00% of $BrdU^+$ cells were also $\beta\text{-Gal}^+$), and these cells expanded, differentiated, and migrated into the GCL over the 14-day observation period (Fig. 6b–h). At the population level, $\beta\text{-Gal}^+$ hippocampal cells initially (day 0) consisted of Type 1 and 2a $Nestin^+/Sox2^+$ NSCs (90.7 ± 1.79%), and a large fraction of $\beta\text{-Gal}^+$ cells were specifically Type 1, radial, $GFAP^+/Sox2^+$ NSCs (53.8 ± 5.49%, Fig. 6b–f). By day 5, the total number of $\beta\text{-Gal}^+$ cells had increased, but to the same extent in Fc-ephrin-B2 and Anti-Fc groups (Supplementary Fig. 5a). At this time point, however, a larger fraction of the $\beta\text{-Gal}^+$ cell population in Fc-ephrin-B2 injected mice had shifted from Type 1 and 2a NSCs to neuronal fate committed Type 2b $DCX^+/Sox2^+$ precursors and even more so to Type 3 $DCX^+/NeuroD1^+$ neuroblasts, as compared to Anti-Fc injected mice (Fig. 6d,e). By day 14, the total number of $\beta\text{-Gal}^+$ cells was again consistent between experimental groups, but the increase in neuronal fated, $\beta\text{-Gal}^+$ cells in Fc-ephrin-B2 versus Anti-Fc injected mice persisted as Type 2b and Type 3 cells matured into $NeuN^+$ GCL neurons (Supplementary Fig. 5a and Fig. 6d–f).

To gain further insights into the early fate decisions that ephrin-B2 modulates,[10] we analyzed individual $\beta\text{-gal}^+$ cell clusters that are likely clonal. This quantification was conducted at day 5 in Nestin/Sox2 co-stained hippocampal sections (a total of 916 and 1145 cell clusters analyzed in n=4 Anti-Fc control and n=5 Fc-ephrin-B2 treated brains, respectively). Type 1 (**1**) and Type 2a (**2a**) cells were identified by $Nestin^+/Sox2^+$ co-staining and distinguished by morphology, and $\beta\text{-gal}^+/Nestin^-/Sox2^-$ cells were deemed astrocytes (**A**) or other neuronal cells (**N**, presumably neuronal precursors, neuroblasts, or mature neurons) by morphology and positioning relative to the GCL (Fig. 6i). We observed that the vast majority of clonal $\beta\text{-gal}^+$ cell clusters contained 3 or fewer cells. Importantly the average number of cells per cluster was 1.55 ± 0.03 and 1.54 ± 0.04 cells in Anti-Fc and Fc-ephrin-B2 sections respectively, again indicating that Fc-ephrin-B2 did not enhance

proliferation of Type 1 or Type 2a cells, in contrast to the previously observed increase in $BrdU^+$ cells with Fc-ephrin-B2 (Fig. 3c). Furthermore, the cell phenotype distribution of the $\beta\text{-gal}^+$ cell clusters mirrored the overall, population-level results (Supplementary Fig. 5b and Fig. 6b–f). Specifically, the clearest result was that Fc-ephrin-B2 induced a significant decrease in the percentage of single Type 1 and single Type 2a cells and a corresponding increase in the number of single N cells (Fig. 6i). Likewise, there was a statistically significant decrease in the overall number of doublets containing a Type 1 cell (1 + X) or a Type 2a cell (2a + X), accompanied by an increase in the number of N + N doublets. These results are consistent with the interpretation that the ephrin-B2 induced the conversion of Type 1 and 2a cells toward more differentiated phenotypes, either with or without a cell division event. Finally, there was no significant change in the percentage of astrocyte-containing clonal $\beta\text{-gal}^+$ cell clusters, as anticipated due to the low levels of gliogenesis previously observed in this experimental paradigm (Supplementary Fig. 1). In summary, these results demonstrate that ephrin-B2 signaling significantly increases the commitment of Type 1 and Type 2a NSCs to a neuronal fate.

Wnt-independent induction of NSC neuronal differentiation

Activation of the canonical Wnt pathway elevates levels of β-catenin, followed by its nuclear translocation and association with Tcf/Lef transcription factors. Recently, this pathway was shown to regulate adult NSC differentiation by transcriptionally activating the proneural transcription factor NeuroD1[23,37]. In zebrafish paraxial mesoderm, ephrin/Eph forward signaling recruits β-catenin to adherens junctions[38], and EphB receptors are known transcriptional targets of β-catenin/Tcf signaling in stem cells in the mammalian gut[39]. However, to our knowledge, ephrin/Eph signaling has not previously been shown to activate β-catenin signaling. Notably, we found that Fc-ephrin-B2 stimulation progressively increased the levels of intracellular active β-catenin in cultured NSCs over a 24 hour period (Fig. 7a), initially detectable at 4 hours and rising to maximal levels by 24 hours. To determine whether this increased active β-catenin was mediated by EphB4, NSCs were pre-incubated with anti-EphB4 antibody prior to Fc-ephrin-B2 addition. As observed in previous experiments (Fig. 2c,4b), EphB4 blockage decreased NSC responses to ephrin-B2 signaling and correspondingly limited the intracellular accumulation of active β-catenin (Fig. 7b).

In the canonical Wnt pathway, intracellular β-catenin levels are directly regulated by glycogen synthase kinase-3 beta (GSK3β), which phosphorylates and thereby marks β-catenin for proteasomal degradation[40]. To determine whether elevated β-catenin activity is required for ephrin-B2's proneuronal effect, we generated NSCs expressing a constitutively active form of GSK3β(GSK3β S9A)[41]. Upon stimulation with Fc-ephrin-B2, both naïve NSCs and NSCs carrying a control empty retroviral vector (CTL NSCs) exhibited increased levels of active β-catenin (Fig. 7c), consistent with prior results (Fig. 7a). However, GSK37S9A NSCs were unable to accumulate β-catenin in response to ephrin-B2 signaling. Furthermore, unlike naïve cells, GSK3 βS9A NSCs resisted neuronal differentiation when stimulated with Fc-ephrin-B2 or co-cultured with ephrin-B2 expressing naïve astrocytes, demonstrating that increased active β-catenin levels are required for ephrin-B2's proneuronal effect (Fig. 7d,e).

These results raise the possibility that ephrin-B2's pro-neuronal effect could involve Wnt ligand mediated activation of β-catenin. To determine whether ephrin-B2 signaling indirectly activates β-catenin via upregulation of soluble Wnts *in vivo*, lentiviruses encoding a Tcf-Luc construct that reports β-catenin activity[42] and either a dnWnt-IRES-GFP[23] (dominant negative Wnt) or IRES-GFP (control) cassette were used. We first confirmed that lentiviral mediated expression of the soluble dnWnt by cultured NSCs significantly decreased luciferase reporter expression upon incubation of the cells with Wnt3a (Supplementary Fig. 6). β-catenin reporter vector, as well as vector expressing dnWnt or GFP alone, was then co-administered *in vivo*. After two weeks, mitotic cells in the rat hippocampi were labeled with BrdU, and Fc-ephrin-B2 or PBS was administered by intrahippocampal injection. After 24 hours, in animals injected with Tcf-Luc and IRES-GFP constructs, Fc-ephrin-B2 increased the number of $Luc^+/BrdU^+$ cells, which also co-stained for active β-catenin (ABC) indicating that ephrin-B2 stimulates β-catenin signaling *in vivo* (Fig. 7f,g). Furthermore, administration of the dnWnt-IRES-GFP vector with the Tcf-Luc vector knocked down the number of $Luc^+/BrdU^+$ cells proportionally in the Fc-ephrin-B2 and PBS injected groups, indicating that Wnt signaling is active in the hippocampus, but that ephrin-B2 still stimulates β-catenin signaling even when Wnt is inhibited. Consistent with these results, dnWnt reduced the number of newborn neurons in both the Fc-ephrin-B2 and PBS injected animals by day 5, but the former still showed a significantly higher number of $DCX^+/BrdU^+$ cells than the latter (Fig. 7g). Thus, ephin-B2 signaling does not apparently activate β-catenin through a soluble Wnt intermediate, and it can increase adult neurogenesis in the absence of Wnt signaling.

Finally, in NSC cultures stimulated with Fc-ephrin-B2, we observed a significant increase in the transcription of both *Mash1* (*Ascl1*) and *Neurod1*, proneural transcription factors previously shown to play roles in adult hippocampal neurogenesis[37,43] (Supplementary Fig. 7a,b). This mRNA upregulation presumably occurs within the subset of cells that undergo neuronal differentiation. Therefore, our collective results strongly indicate that hippocampal astrocytes instruct neuronal differentiation of $EphB4^+$ NSCs through juxtacrine ephrin-B2/EphB4 forward signaling, which induces the expression of proneural transcription factors via a β-catenin-dependent and soluble Wnt independent mechanism.

Discussion

Stem cell niches present repertoires of signals that control cell maintenance, proliferation, and differentiation. Recent studies have identified several factors that regulate cell maintenance and proliferation within the adult NSC niche; however, few cues have been found to induce differentiation. Furthermore, whereas numerous soluble cues have been found to regulate adult neurogenesis, cell-cell interactions in the niche have in general been less studied. We have found that ephrin-B2 presented from hippocampal astrocytes activates β-catenin signaling in NSCs, upregulates the expression of key proneural transcription factors, and instructs their neuronal differentiation (Supplementary Fig. 8).

Hippocampal astrocytes, but not astrocytes derived from non-neurogenic regions of the central nervous system, regulate neurogenesis[22], and a recent expression profiling analyzed the differential expression of factors that underlie this activity[19]. However, ephrin-B2 was

not represented on the Affymetrix chip used for this important comparative gene expression study, and cues dependent on cell-cell contact between the stem cells and hippocampal astrocytes have not been explored. The importance of ephrins and their receptors in axon guidance, neural tissue patterning, and synapse formation is well established[24]; however, less is known about their role in regulating adult NSCs. Several studies have shown that ephrin/Eph signaling can affect NSC proliferation[31–33,44]; however, these important studies did not address the potential for ephrins and Ephs to regulate stem cell differentiation within the brain. This work thus represents the first case of ephrin/Eph-family regulation of NSC neuronal lineage commitment in the adult CNS.

We have shown that endogenous ephrin-B2 is expressed by astrocytes in close proximity to adult NSCs, shRNA-mediated knockdown of the endogenous ephrin-B2 substantially lowers the fraction of newborn cells that become DCX+ neuronal precursors, and exogenous addition of ephrin-B2 induces the conversion of Type 1 and 2a NSCs toward Type 2b precursors and subsequently neurons (Fig. 2,5,6). Furthermore, lineage tracing analysis revealed a decrease in single Type 1 and Type 2a cells and an increase in the number of single neuroblasts and neurons in the presence of ephrin-B2, as well as a decrease in the number of cell doublets containing a Type 1 or 2a cell with a corresponding increase in the number of neuroblast and neuron doublets (Fig. 6). These results are consistent with ectopic ephrin-B2 inducing β-catenin signaling and upregulation of proneural transcription factors (Fig. 7 and Supplementary Fig.7a,b) to induce NSC differentiation independent of cell division. Collectively, our results indicate that juxtacrine signaling between astrocytes and NSCs provides a mechanism for the niche to locally control NSC differentiation. Prior results have investigated the importance of Notch and EphB2 signaling in modulating the properties of neighboring cells in the NSC niche[45] and the importance of Notch in maintaining Type 1 NSCs[13,46]. Together, these results increasingly establish cell contact-dependent signaling as a critical mechanism for locally regulating multiple stages in adult neurogenesis.

Ephrin/Eph signaling is generally known to be bidirectional, such that the Eph-presenting cell can activate signaling within the ephrin ligand-expressing cell[24]. We find that adult NSCs significantly upregulate ephrin-B2 and downregulate EphB4 expression upon differentiation into astrocytes *in vitro*, yet retain EphB4 expression upon neuronal differentiation (Supplementary Fig. 2b). Ephrin/Eph signal feedback from the differentiating NSC to neighboring cells could thus represent a mechanism to dynamically remodel the signaling environment of the niche, analogous to GDF11-dependent negative feedback from neurons to neural progenitors in the olfactory epithelium[47] or EGF-dependent feedback from neural progenitors to neural stem cells in the SVZ[48]. In addition, ephrin and Eph expression dynamics could help control cell differentiation following NSC symmetric or asymmetric division[8] and thereby contribute to maintaining or modulating the cellular composition of the niche. The potential of ephrin signaling in general to support self-renewing, asymmetric cell division – i.e. to generate a stem cell and a differentiated progeny – is virtually unexplored, as there is only one report of ephrin mediated regulation of asymmetric stem cell division, in the ascidian embryo[49].

Type 1 NSCs were depleted upon ephrin-B2 administration (Fig. 6), indicating that they may be direct targets of this ligand's signaling. However, while some type 1 cells expressed levels of EphB4 detectable by immunostaining and upregulated β-catenin signaling upon ephrin-B2 addition, nearly all type 2a cells did so, and additional work will be needed to determine whether the observed differentiation of type 1 cells in response to ephrin-B2 is direct and/or indirect. In addition, while ephrin-B2 knockdown did not impact cell proliferation (Fig. 5d), and ephrin-B2 protein administration did not affect the proliferation of genetically-labeled, Nestin-expressing stem and progenitor cells (Fig. 6 and Supplementary Fig. 5), ephrin-B2 addition did result in the expansion of cells pre-labeled with BrdU in the rat brain. It is thus conceivable that in addition to its clear role in inducing the differentiation of NSCs at the expense of stem cell maintenance or self-renewing cell divisions (Fig. 6), ephrin-B2 could also modulate the expansion of later stage neuroblasts that are strongly labeled with BrdU, a possibility that could be explored in future work.

In summary, our findings reveal ephrin-B2, a transmembrane factor known for its role in cell and tissue patterning, as a key regulator of adult hippocampal neurogenesis, the first known function of an Eph-family protein in regulating neuronal lineage commitment of NSCs in the adult CNS. Additionally, hippocampal astrocytes are the source of the ephrin-B2 signal, which further supports the emerging view that astroglia are active and essential regulators of an increasing number of adult CNS functions, including remodeling the neurogenic niche through local cellular interactions. Moreover, the discovery that ephrin-B2 signals through β-catenin adds further understanding to the interconnected and likely synergistic nature by which niche factors regulate adult neurogenesis. Finally, this work may have future applications in modulating NSC function for treating brain injury and neurodegenerative disease.

Methods

Cell culture

NSCs isolated from the hippocampi of 6-week-old female Fisher 344 rats (Charles River), were cultured as previously described[14] on poly-ornithine/laminin-coated plates in DMEM/F12 medium (Life Technologies) containing N2 supplement (Life Technologies) and 20 ng/mL FGF-2 (PeproTech), with subculturing upon reaching 80% confluency using Accutase (Phoenix Flow Systems). To induce differentiation, NSCs were cultured for five days in DMEM/F12/N2 medium supplemented with 2% fetal bovine serum (FBS, Life Technologies) and 1 μM retinoic acid (BIOMOL). Rat hippocampal astrocytes were isolated from Fisher 344 rats (Charles River) as previously described[22] and cultured on poly-ornithine/laminin-coated plates in DMEM/F12/N2 supplemented with 10% FBS, with subculture upon reaching 90% confluency using Trypsin EDTA (Mediatech, Inc.).

Fc-ephrin-B2 synthesis and differentiation assays

To generate Fc-ephrin-B2, mouse ephrin-B2/Fc (Sigma-Aldrich) was incubated at a 9:1 ratio (w/w) with a goat, anti-human IgG Fc antibody (Jackson ImmunoResearch USA) for 90 min at 4°C before immediate use. To differentiate NSC *in vitro*, eight-well chamber slides were seeded with 5×10^4 cells per well in standard culture medium containing 20 ng/mL FGF-2.

Next day, the medium was replaced with DMEM/F12 containing 0.5 ng/mL FGF-2 and various concentrations of Fc-ephrin-B2. Differentiation experiments were performed over a 4 day period with a 50% media change daily. Then, the cells were fixed using 4% paraformaldehyde (PFA, Sigma Aldrich) and stained using standard protocols (see below). To test the effect of Eph receptor blocking, NSCs were pre-incubated with goat anti-EphB4 or anti-EphB2 (1:50, Santa Cruz Biotechnology) for 30 minutes before addition of Fc-ephrin-B2 ligands.

Lentiviral and retroviral vector construction

DNA cassettes containing either human or mouse U6 promoter driven expression of candidate shRNAs against rat *efnb2* (Gene ID: 306636) were constructed by PCR. The forward primer containing a *Pac* I site for cloning and the reverse primer containing the entire shRNA sequence were used to amplify the U6 promoter from template plasmids pFhU6ABCgUGW (unpublished) or pmU6 pro. PCR was performed using Phusion high fidelity polymerase (Finnzymes) under the following conditions: pre-incubation at 98°C for 2 min; 30 cycles, with 1 cycle consisting of 12 s at 98°C, 30 s at 55°C – 65°C, and 15 s – 25 s at 72°C; and the final extension step of 2 min at 72°C. The U6 sense primers were sense_hU6 (5'-AACAATTAATTAAAAGGTCGGGCAGGAAGAGGGCCTATT-3') and sense_mU6 (5'-AACAATTAATTAAATCCGACGCCGCCATCTCTAGGCC-3'). For a listing of the shRNA encoding antisense primers see Supplementary Table 1. In parallel, a control shRNA cassette against *LacZ* was constructed[50] analogously, using pBS U6 shRNA β-gal as the template and with primers listed in Supplementary Table 1. All PCR products were digested with *Pac* I and cloned into the pFUGW lentiviral vector[51] upstream of the human ubiquitin-C promoter and eGFP. pCLGPIT-GSK3 βS9A encoding a constitutively active form of GSK3 β (a gift of Ashley Fritz and Smita Agrawal) was constructed by amplifying the GSK3 βsequence from rat NSC cDNA, inserting it into pCLGPIT, and subjecting the plasmid to a site-directed mutagenesis to introduce the S9A mutation. Lentiviral and retroviral vectors were packaged using standard methods as described elsewhere[52,53]. Lastly, to validate functionality of the *efnb2* shRNA vectors, the mouse GFAP promoter[54] as well as the human GFAP promoter[55] was cloned into the digested pFUGW vector, replacing the ubiquitin promoter upstream of eGFP.

Ephrin-B2 RNAi co-cultures

To select effective shRNA sequences targeting *efnb2*, hippocampus-derived astrocytes were transduced with lentivirus encoding trial shRNA sequences at an MOI of 3 and cultured for four days. RNA isolated from astrocyte cultures was analyzed for *efnb2* expression using QPCR (see below and Supplementary Figure 3). For co-culture assays, hippocampus-derived astrocytes were transduced with lentivirus encoding shRNA sequences at an MOI of 3 and cultured for two days prior to a 3 day incubation in medium containing 20 μM cytosine arabinoside (AraC, Sigma-Aldrich) to deplete rapidly dividing astrocytes. Then, cells were returned to standard medium for 24 hours, before being subcultured onto 8-well chamber slides at a density of 70,000 cells/cm^2. NSCs were transduced with retrovirus encoding either pCLGPIT or pCLGPIT-GSK3 βS9A at an MOI of 1 and cultured for two days before a 96-hour selection period in medium containing 1 μg/mL puromycin (Sigma-Aldrich). Next, NSCs were incubated in standard media containing 25 μM

bromodeoxyuridine (BrdU, Sigma-Aldrich) for 48-hours. Then, the BrdU-labeled NSCs were seeded on top of a monolayer of astrocytes in 8-well chamber slides at a density of 70,000 cells/cm^2 in medium lacking FGF-2. Co-cultures were maintained for six days prior to fixation and analysis by immunocytochemistry.

In vivo gain and loss of function studies

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California Berkeley. Eight-week-old adult female Fisher 344 rats received daily 50 mg/kg intraperitoneal (i.p.) injections of BrdU (Sigma Aldrich) dissolved in saline to label mitotic cells, as previously described[14]. In Fc-ephrin-B2 studies, animals were anesthetized prior to 3 μL bilateral intrahippocampal stereotaxic injections of either PBS (Life Technologies), ephrin-B2 (14 μg/mL), Anti-Fc antibody (126 μg/mL), or Fc-ephrin-B2 (140 μg/mL) in PBS. The injection coordinates were −3.5 mm anteriorposterior and ± 2.5 mm mediolateral relative to bregma, and −3.0 mm dorsoventral relative to dura. Refer to Figure 3a for injection time course. Unbiased stereology (Zeiss Axio Imager, software by MicroBrightfield) using the optical fractionator method was performed on eight, evenly distributed sections from each rat to estimate the total number of relevant cells throughout the entire hippocampus. In RNAi studies, rats received bilateral intrahippocampal injections of 3 μL of lentiviral solutions in PBS on day −19. The injection coordinates with respect to bregma were −3.5 mm anteriorposterior, −3.5 mm dorsoventral (i.e. from the dura), and ± 2.0 mm mediolateral. Refer to Figure 5a for injection time course. Unbiased stereology was performed on eight GFP$^+$ hippocampal sections from each rat, and the number of selected cells was normalized by the volume of hippocampal tissue analyzed.

In vivo fate mapping

Nestin-CreER^{T2} mice[35] (a kind gift from Amelia Eisch, UT Southwestern, Dallas, TX) and *R26-stop$^{fl/fl}$-lacZ* mice[36] (a kind gift from John Ngai, UC Berkeley, Berkeley, CA), both having a 100% C57/Bl6 background, were crossbreed twice to generate a homozygous *Nestin-CreER^{T2};R26-stop$^{fl/fl}$-lacZ* mosue strain. Geneotyping was performed using the following primers: Cre-F (5'-ACCAGCCAGCTATCAACTCG-3'), Cre-R (5'-TTACATTGGTCCAGCCACC-3'), 200bp; LacZ-F (5'-GTCAATCCGCCGTTTGTTCCCACG-3'), LacZ-R (5'-CCAGTACAGCGCGGCTGAAATCAT-3'), 400 bp; wtRosa-F (5'-GGAGCGGGAGAAATGGATATG-3'), wtRosa-R (5'-AAAGTCGCTCTGAGTTGTTAT-3'), 600bp. To induce recombination and label mitotic cells, 5 week old *Nestin-CreER^{T2};R26-stop$^{fl/fl}$-lacZ* mice were administered i.p. 180 mg/kg tamoxifen (SigmaAldrich) dissolved in corn oil and 50 mg/kg BrdU daily for three days prior to intrahippocampal injections of experimental solutions. 1 μL of Anti-Fc (126 μg/mL) and Fc-ephrin-B2 (140 μg/mL) in PBS were then administered at −2.12 mm anteriorposterior, −1.55 mm dorsoventral (from dura), and ± 1.5 mm mediolateral with respect to the bregma. Pre-determined groups of mice (balanced in male-to-female ratio) were sacrificed at Day 0 (Sham), 5, and 14 time points, and tissue collection and histology were performed.

In vitro validation of β-catenin reporter and dnWnt vectors

NSCs were co-infected with 7xTcf-FFluc[42] (Tcf-Luc) and either LV-dnWnt-IRES-GFP[23] or LV-GFP and expanded in culture. After a 24-hour pulse with Wnt3a (200 ng/mL), cell lysates were collected and analyzed using Luc-Screen® Extended-Glow Luciferase Reporter Gene Assay System (Applied Biosystems) and a TD-20/20 luminometer (Turner BioSystems) to determine the relative level of β-catenin activation.

In vivo β-catenin activation and Wnt-independent neurogenesis

Eight-week-old adult female Fisher 344 rats received bilateral, intrahippocampal, stereotaxic injections of 3 μL of a mixture of half 7xTcf-FFluc[42] (Tcf-Luc) and half LV-dnWnt-IRES-GFP[23] or LV-GFP (kind gifts from Fred Gage, Salk Institute, La Jolla, CA) lentiviral vectors on Day −17. Starting on Day −3, animals were intraperitoneally injected with BrdU (50 mg/kg) for three days prior to subsequent bilateral intrahippocampal stereotaxic injections of 3 uL of either Fc-ephrin-B2 (140 μg/mL) or PBS on Day 0. The injection coordinates with respect to the bregma were −3.5 mm anteriorposterior, −3.4 mm dorsoventral (from dura), and ± 1.8 mm mediolateral. Animals were sacrificed on Day 1 and half were sacrificed on Day 5 before brains were processed for histology and analyzed by stereology.

Immunostaining and imaging

Cells cultures were fixed with 4% paraformaldehyde (PFA) for 10 minutes, blocked for 1 hour with 5% donkey serum (Sigma), permeabilized with 0.3% Triton X-100 (Calbiochem), and incubated for 48 hours with combinations of the following primary antibodies: mouse anti-nestin (1:1000, BD Pharmingen), rabbit anti-GFAP (1:250, Abcam), rabbit anti- βIII-Tubulin (1:250, Sigma), and rabbit anti-MBP (1:500, Santa Cruz). Appropriate Cy3-, Cy5-, or Alexa Fluor 488-conjugated secondary antibodies were used to detect primary antibodies (1:250, Jackson Immunoresearch; 1:250, Molecular Probes). TO-PRO-3 (10 μM, Life Technologies) or DAPI was used as the nuclear counterstain.

Animals were perfused with 4% PFA (Sigma), and brain tissue was extracted, stored in fixative for 24 hours, and allowed to settle in a 30% sucrose solution. Brains were coronally sectioned and immunostained using previously published protocols[14]. Primary antibodies used were mouse anti-BrdU (1:100, Roche), mouse anti-NeuN (1:100, Millipore), rat anti-BrdU (1:100, Abcam), goat anti-doublecortin (1:50, Santa Cruz Biotechnology), rabbit anti-GFAP (1:1000, Abcam), guinea pig anti-doublecortin (1:1000, Millipore), goat anti-ephrin-B2 (1:10, R&D Systems), rabbit anti-Sox2 (1:250, Millipore), goat anti-EphB4 (1:50, Santa Cruz), mouse anti-GFAP (1:2000, Advanced Immunochemical), rabbit anti-GFP (1:2000, Life Technologies), goat anti-GFP (1:200, Abcam), and rabbit anti- β-Gal (Gift from John Ngai). Appropriate Cy3-, Cy5-, or Alexa Fluor 488-conjugated secondary antibodies (1:125, Jackson Immunoresearch; 1:250, Life Technologies) were used. For sections stained with rat anti-BrdU, biotin-conjugated anti-rat IgG (1:250, Jackson Immunoresearch) was used as the secondary, which was then washed and incubated with Cy3-conjugated streptavidin (1:1000, Jackson Immunoresearch USA) for 2 hours to amplify the signal. DAPI (50 μg/mL, Invitrogen) was used as a nuclear counterstain. Sections were mounted on glass slides and analyzed using the optical fractionator method in unbiased stereological microscopy (Zeiss

Axio Imager, software by MicroBrightfield) and/or imaged with either a Leica Microsystems confocal microscope or a Zeiss 510 Meta UV/VIS confocal microscope located in the CNR Biological Imagining Facility at the University of California Berkeley.

Western blotting

NSCs were seeded at 2.5×10^5 cells per well in a 6-well culture dish in standard culture medium containing 0.1 μg/mL FGF2. The following day, 10 μg/mL Fc-ephrin-B2 was added using a 50% media change. To test the effect of inhibition of the EphB4 receptor, NSCs were pre-incubated with goat anti-EphB4 (1:50, Santa Cruz Biotechnology) for 30 minutes before addition of Fc-ephrin-B2. Cell lysates were collected at various time intervals (0–24 hours after Fc-ephrin-B2 addition) and Western blotted as previously described[56]. On nitrocellulose membranes, proteins were labeled using anti-active β-catenin (1:500, Millipore) and rabbit anti-total GSK-3 β(1:1000, Cell Signaling) primary antibodies in combination with the appropriate HRP-conjugated secondary antibody (1:10,000, Pierce). SuperSignal West Dura Extended Duration Substrate (Pierce) was used to detect the protein bands, and after film development (Kodak Film Processor 5000RA), membranes were stripped and re-probed with rabbit anti-GAPDH (1:2500, Abcam).

RNA isolation and QPCR

RNA samples were isolated using standard Trizol (Life Technologies) collection and ethanol precipitation. RNA samples were quantified using a NanoDrop Spectrophotomer ND-1000 (NanoDrop Technologies, Inc.), and equivalent amounts of RNA were added to QPCR reactions. Quantitative PCR was performed to probe for expression of nestin, GFAP, β-Tubulin III, and 18S mRNA using prior protocols[57]. For analysis of ephrin-B2 mRNA levels, QPCR was performed using a Taqman Gene Expression Assay (Applied Biosystems) for *efnb2* (#Rn01215895_m1) and for eukaryotic *18S* (#803026). All reactions were performed using a BioRad IQ™5 Multicolor Real-Time Detection System, and target mRNA expression levels were normalized according to levels of 18S.

Statistical analysis

Statistical significance of the results was determined using an ANOVA and multiple means comparison function (i.e. Tukey-Kramer Analysis) in MATLAB. Data are represented as means ± s.d.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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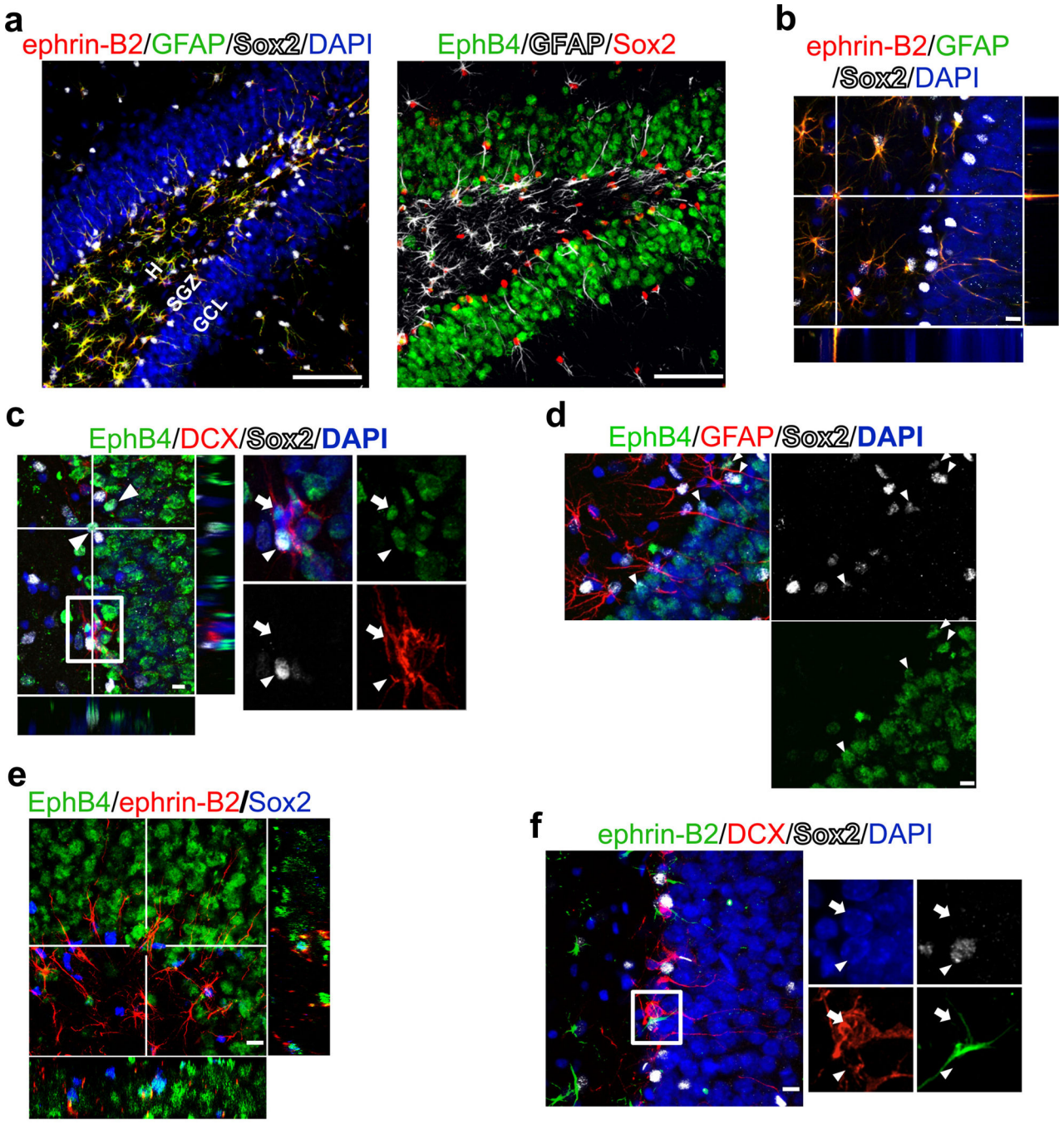

Figure 1.

In vivo, SGZ Type 2a NSCs, Type 2b neuronal precursors, and Type 3 neuroblasts express EphB4, and hippocampal astrocytes express ephrin-B2. (**a**) Staining of the hippocampal dentate gyrus showed that GFAP$^+$ hilar (H) astrocytes express ephrin-B2. In addition, cells in the SGZ and neurons in the granule cell layer (GCL) express EphB4$^+$ on the cell soma. Scale bar represents 100 μm. (**b**) GFAP$^+$ astrocytes adjacent to the SGZ co-express ephrin-B2. (**c,d**) EphB4 expression persists throughout NSC neuronal differentiation, including Type 2a NSCs (Sox2$^+$/DCX$^-$/GFAP$^-$), Type 2b neuronal precursors (Sox2$^+$/DCX$^+$), and

Type 3 neuroblasts (DCX^+). (**c**) Confocal images show EphB4 expression in $Sox2^+/DCX^-$ cells (large arrowheads) in the SGZ. Magnified region depicts the presence of EphB4 expression on a $Sox2^+/DCX^+$ Type 2b neuronal precursor (small arrowhead) and a DCX^+ Type 3 neuroblast (arrow). (**d**) Confocal images also show EphB4 expression by $Sox2^+/GFAP^-$ cells (small arrowheads); therefore, $Sox2^+/DCX^-/GFAP^-$ Type 2a NSCs also express EphB4. (**e**) Given the close proximity of ephrin-$B2^+$ astrocytes to $EphB4^+$ cells in the SGZ, ephrin-B2/EphB4 juxtacrine signaling is in a position to induce NSC differentiation into (**f**) $Sox2^+/DCX^+$ Type 2b neuronal precursors (small arrowhead) and subsequently $Sox2^-/DCX^+$ Type 3 neuroblasts (arrow). The scale bars represent 10 μm.

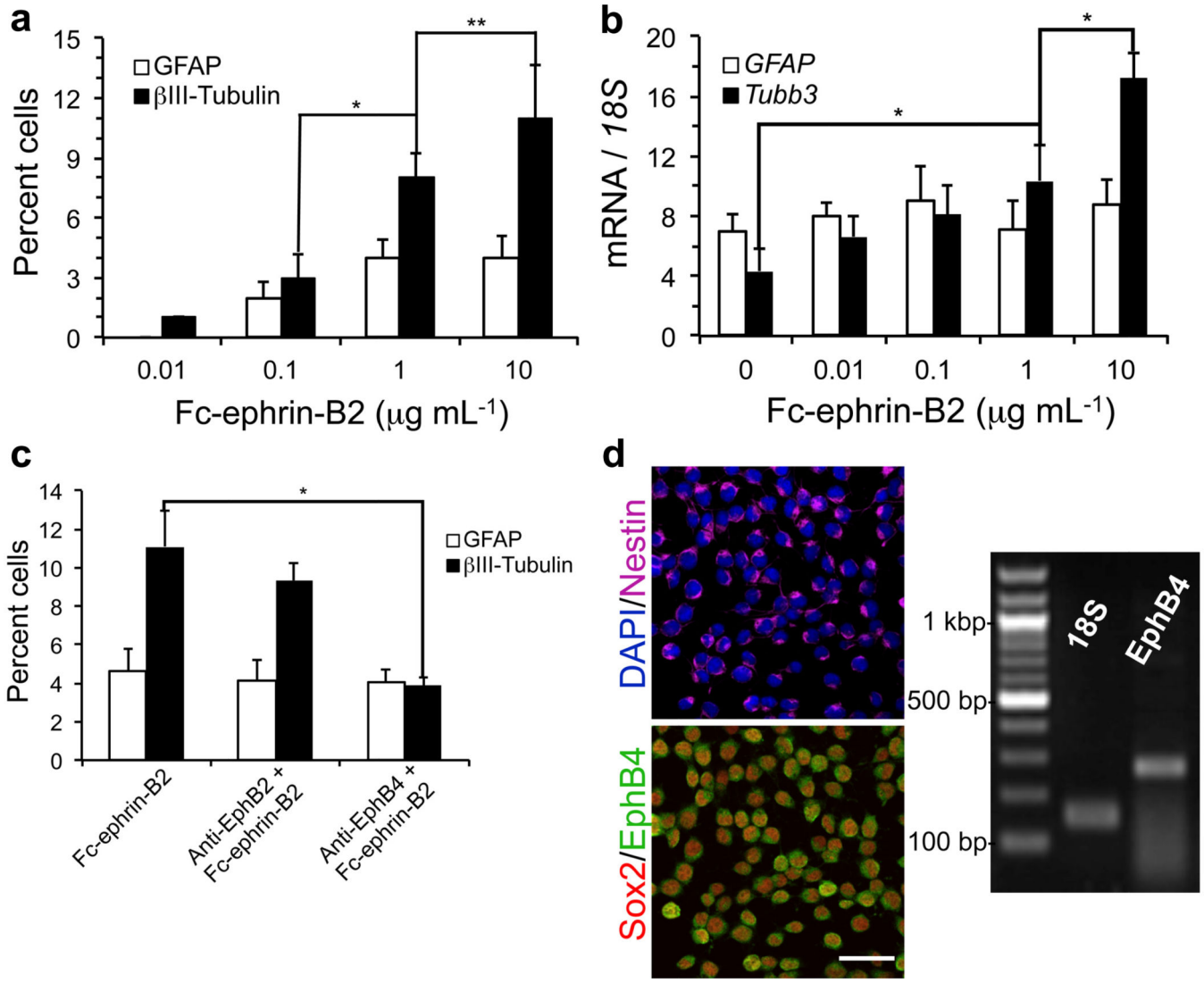

Figure 2.

Fc-ephrin-B2 promoted the neuronal differentiation of NSCs *in vitro*. (**a,b**) Stimulation by Fc-Ephrin-B2 induced NSCs to undergo neuronal differentiation (βIII-Tubulin$^+$ or *Tubb3*) in a dose-responsive fashion as measured by immunocytochemistry and QPCR (n = 3, experimental replicates). Glial fibrilary acidic protein (GFAP) staining was slightly increased with ephrin-B2, but no increase in expression was observed by QPCR. (**c**) Blockage of ephrin-B2 receptors, EphB2 and EphB4, during Fc-ephrin-B2 (10 μg/mL) stimulation revealed that EphB4 mediates Fc-ephrin-B2's proneuronal effect on NSCs (n = 3, experimental replicates). ANOVA plus a multi-variable Tukey-Kramer analysis was conducted, with * indicating P <0.01 and ** indicating P <0.05. Data are represented as means ± s.d. (**d**) Nestin$^+$/Sox2$^+$ NSCs express EphB4 as demonstrated by immunocytochemistry and RT-PCR. Scale bar represents 100 μm.

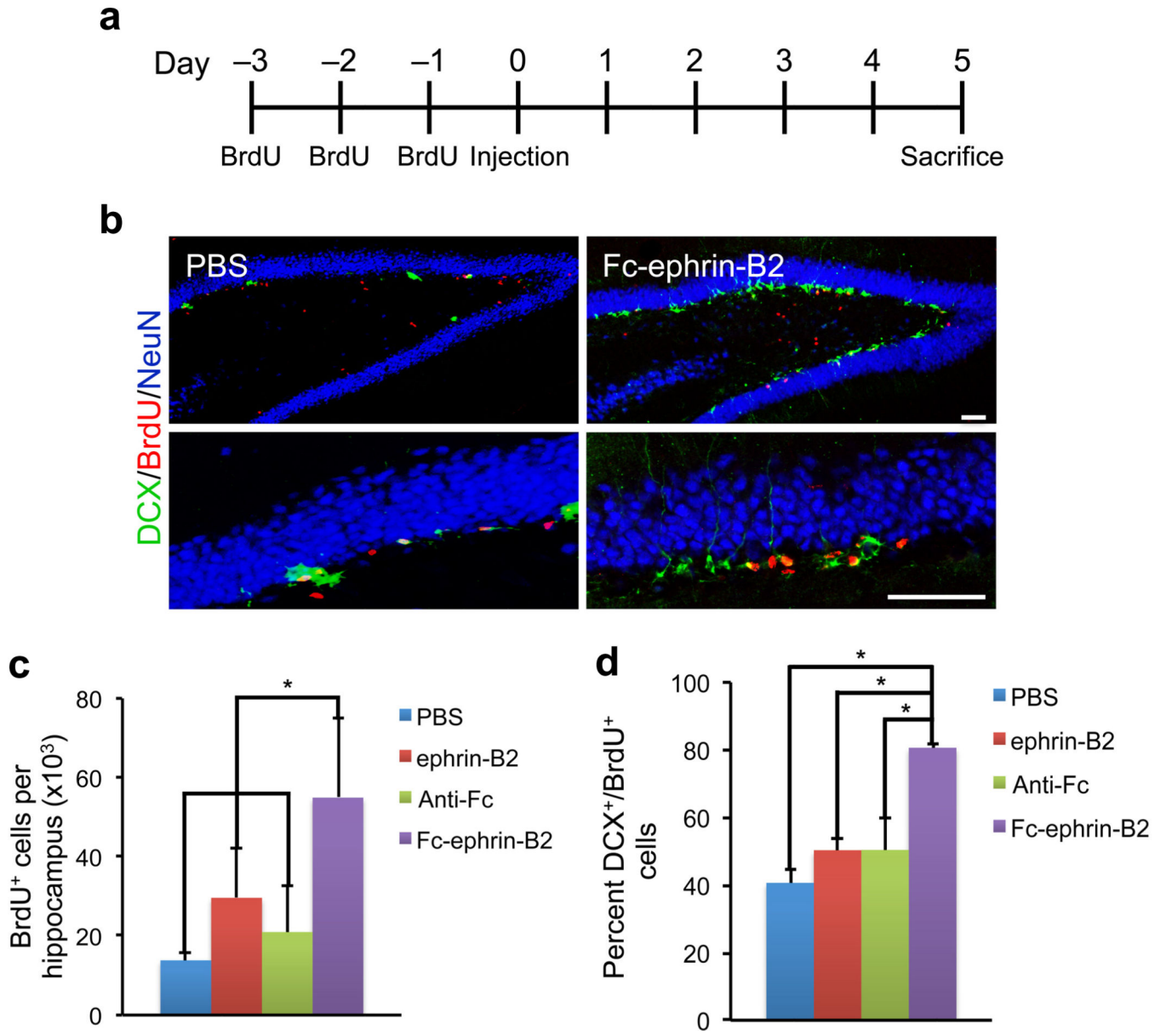

Figure 3.

Intrahippocampal injection of Fc-ephrin-B2 increases neurogenesis in the SGZ. (**a**) Schematic of experimental time course. (**b**) Administration of Fc-ephrin-B2 into the hippocampus significantly increased neurogenesis as shown by representative confocal images of the dentate gyrus and SGZ (close-up). The scale bar represents 100 μm. (**c**) The injection of exogenous ephrin-B2 ligands increased $BrdU^+$ cell numbers compared to vehicle and constituent controls. (**d**) Only injection of Fc-ephrin-B2, but not its constituent controls, increased the percentage of $BrdU^+$ cells that co-stained for DCX in the SGZ (n = 3 brains, analyzed 8 hippocampal sections per brain). * indicates P <0.01, and ** indicates P <0.05; ± s.d.

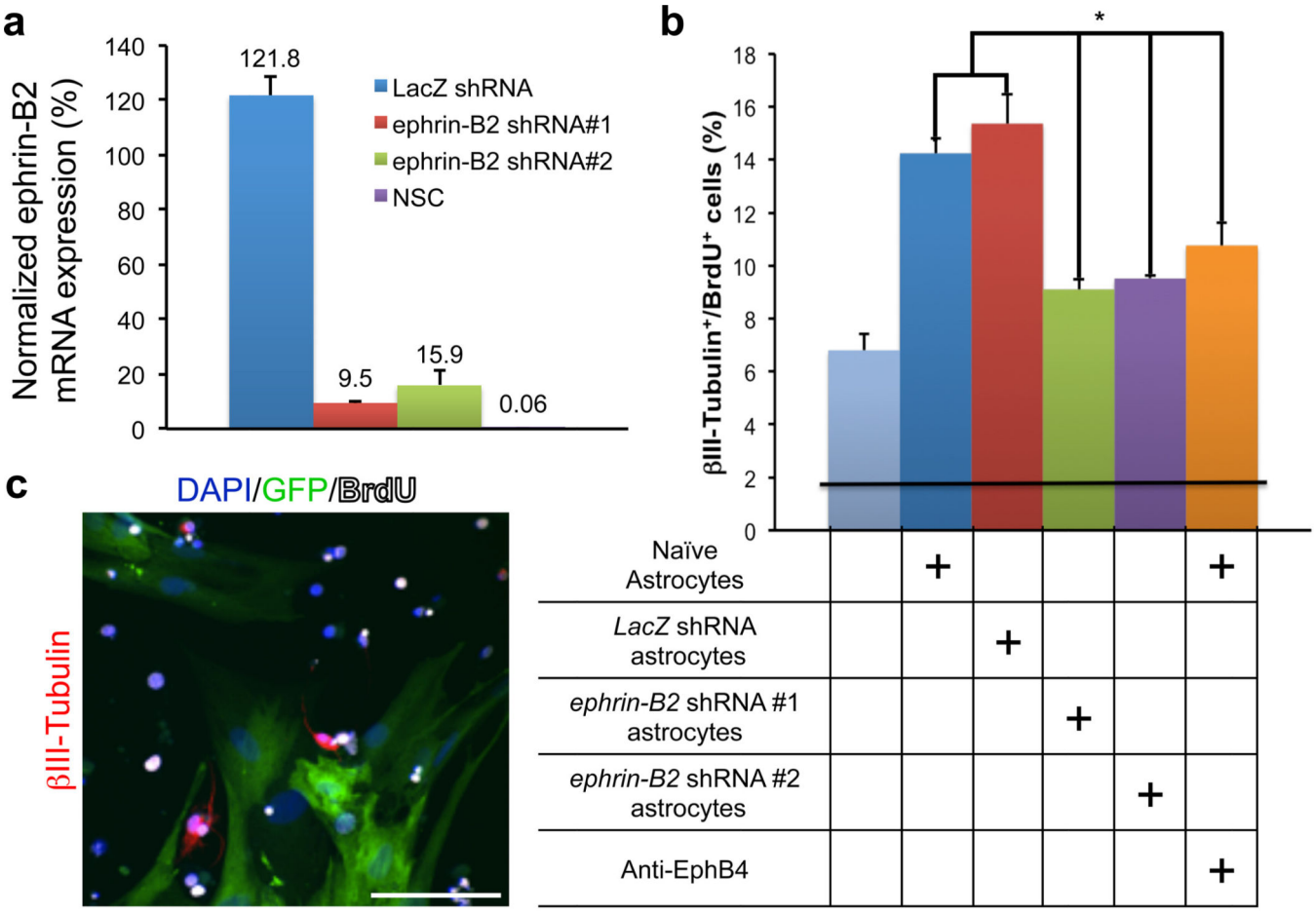

Figure 4.

Ephrin-B2 RNAi decreases the proneuronal effect of hippocampus-derived astrocytes *in vitro*. (**a**) *Efnb2* shRNA lentiviral vectors (#1 and #2) significantly inhibit *efnb2* expression in hippocampus-derived astrocytes. Expression levels were measured by QPCR and normalized to *efnb2* expression in non-infected hippocampus-derived astrocytes (i.e. 100%). Also, hippocampus-derived astrocytes express orders of magnitude more *efnb2* than NSCs (n = 3, technical replicates). (**b**) Naïve astrocytes and astrocytes expressing a control *LacZ* shRNA promoted neuronal differentiation of NSCs, compared to NSC-only cultures. However, knockdown of astrocyte *efnb2* expression, or antibody blockage of NSC EphB4 receptors, significantly diminished the proneuronal effect of hippocampus-derived astrocytes, as demonstrated by the decrease in the percentage of βIII-Tubulin$^+$/ BrdU$^+$ NSCs to levels closer to those in NSC-only cultures (n = 4, experimental replicates). The solid black line indicates the level of βIII-Tubulin$^+$/BrdU$^+$ cells at the start of the experiment. (**c**) Representative confocal image of BrdU-labeled NSCs differentiated into βIII-Tubulin$^+$ neurons after co-culture with lentiviral vector-expressing, i.e. GFP$^+$, astrocytes. NSCs adjacent to astrocytes had a higher propensity for neuronal differentiation. Scale bar represents 100 μm. * indicates P <0.01; ± s.d.

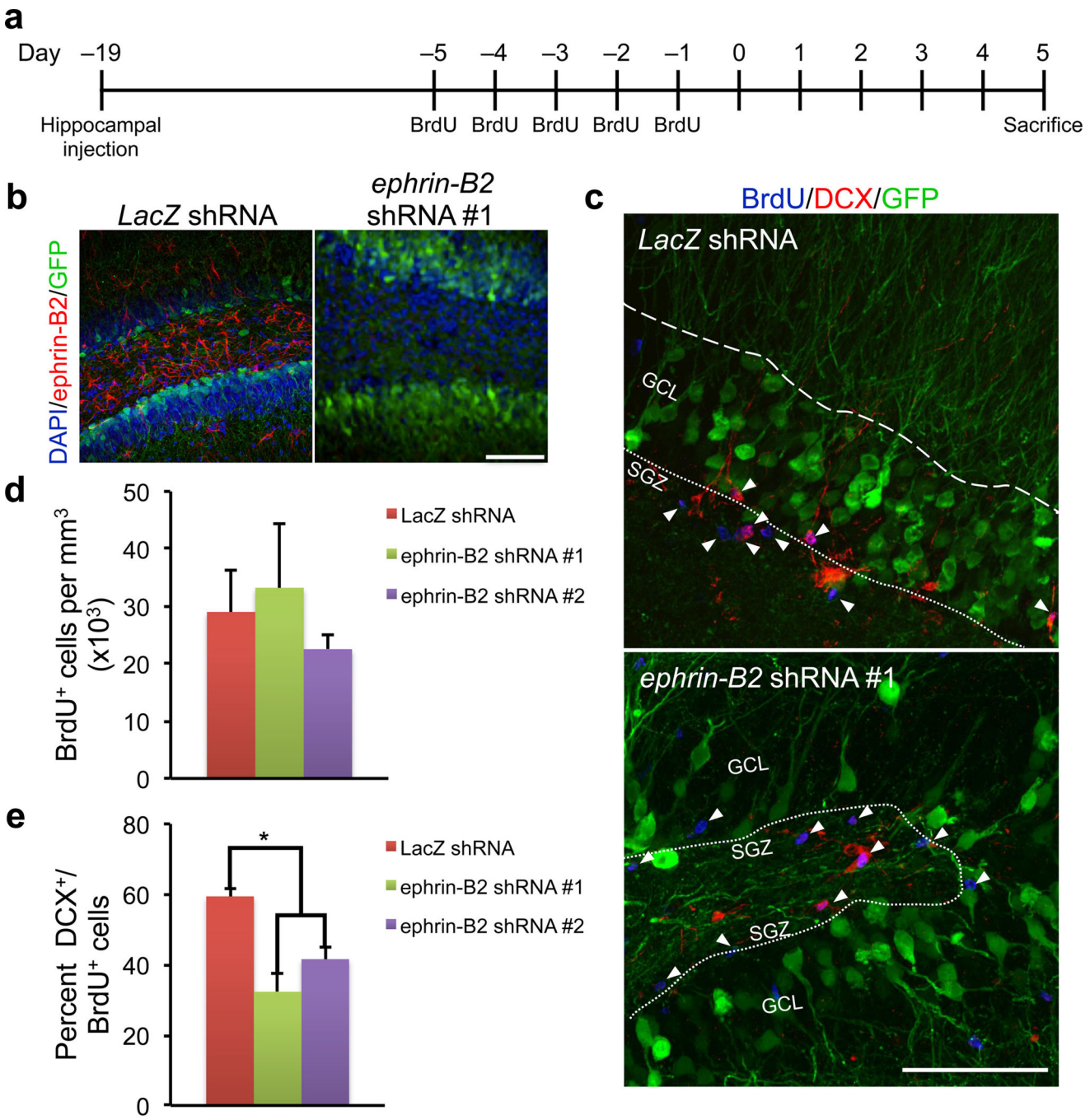

Figure 5.

Ephrin-B2 RNAi decreases neuronal differentiation of $BrdU^+$ cells in the SGZ. (**a**) Schematic of experimental time course. (**b**) Regions of the hippocampus transduced with lentiviral vector (GFP^+) carrying *efnb2* shRNA #1 and #2 (data not shown) showed considerably less ephrin-B2 staining than hippocampi transduced with *LacZ* shRNA lentivirus injected rats. (**c**) Representative confocal images showing decreased neuronal differentiation, i.e. DCX^+ co-staining, of $BrdU^+$ (arrowheads) cells in the SGZ of rats injected with lentivirus encoding *efnb2* shRNA. Scale bars represent 100 μm. (**d,e**)

Knockdown of ephrin-B2 in the hippocampal niche did not affect $BrdU^+$ cell numbers, but it did result in a significant decrease in the percentage of $BrdU^+$ cells that co-stained for DCX in the SGZ (n = 4 brains, analyzed 8 hippocampal sections per brain). This suggests that endogenous ephrin-B2 signaling regulates neuronal differentiation of NSCs. * indicates $p<0.01$; ± s.d; dotted line marks SGZ/Hilus boundary and dashed line marks GCL/MCL boundary.

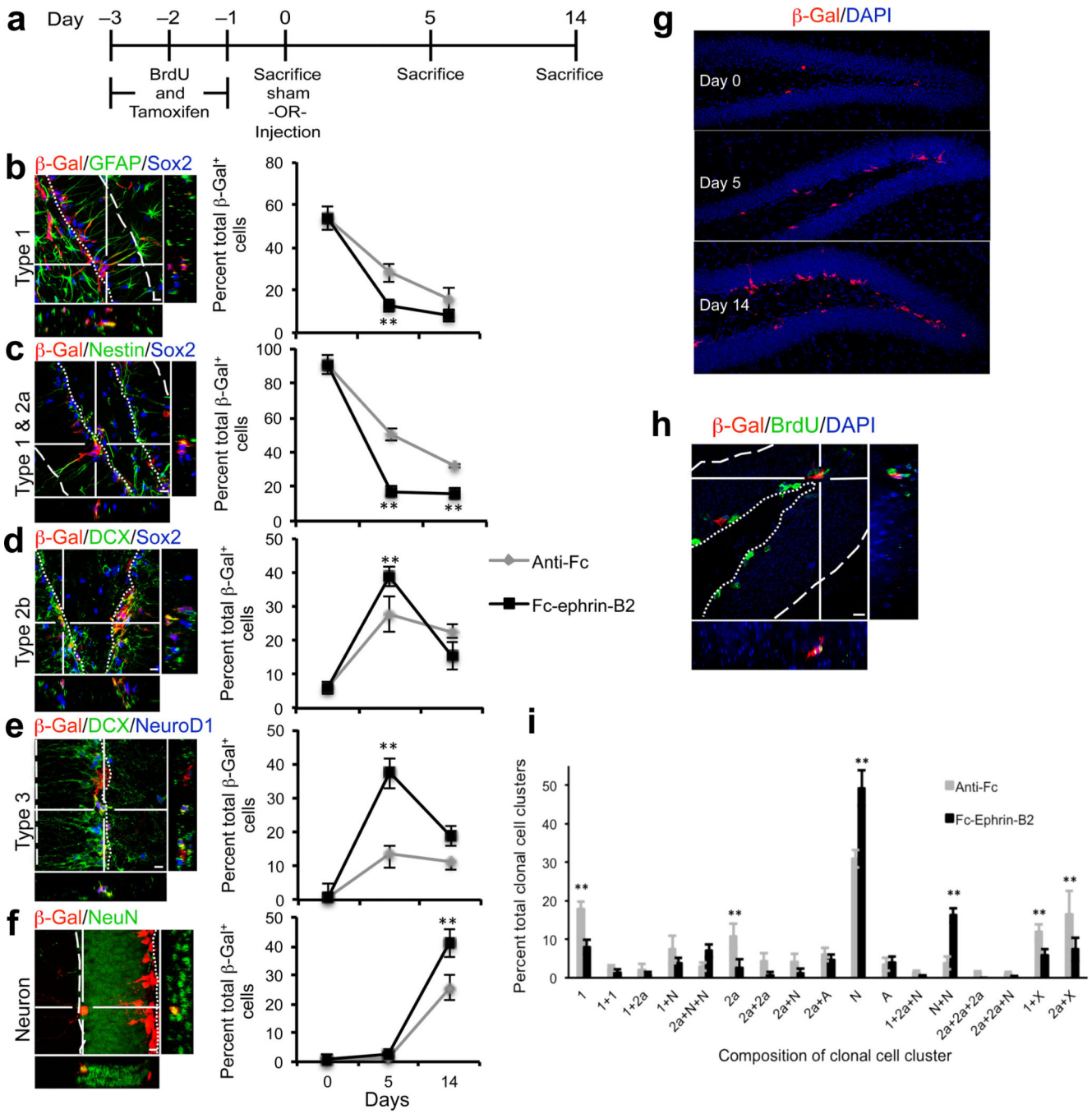

Figure 6.

Lineage tracing of ephrin-B2-induced NSC differentiation. (**a**) Time course where (**b,c**) initially 90.7 ± 1.79% of β-Gal$^+$ cells were Nestin$^+$/Sox2$^+$, and 53.8 ± 5.49% were GFAP$^+$ along radial process (Type 1 NSCs). (**d,e**) By day 5, 38.7 ± 2.73% and 37.3 ± 4.51% of β-Gal$^+$ cells were Sox2$^+$/DCX$^+$ and DCX$^+$/NeuroD1$^+$, respectively, in Fc-ephrin-B2 injected mice vs. 27.7 ± 5.16% and 13.8 ± 4.38% in Anti-Fc controls. (f) At day 14, 41.1 ± 4.86% of β-Gal$^+$ cells were NeuN$^+$ for Fc-ephrin-B2 vs. 25.6 ± 4.24% for controls. (**g,h**) Representative sections where β-Gal$^+$ and BrdU$^+$ cells proliferated and differentiated over

14 days. (i) Compared to Anti-Fc, Fc-ephrin-B2 decreased the number of single Type 1 (“1”, 8.01 ± 1.91% vs. 17.8 ± 1.93%) and Type 2a NSCs (“2a”, 2.51 ± 2.34% vs. 10.8 ± 3.22%) and increased single neuroblast or neurons (“N”, 49.2 ± 4.57% vs. 30.9 ± 2.28%). Furthermore, ephrin-B2 decreased the number of doublets containing a Type 1 (“1+X”, 5.95 ± 1.47% vs. 11.8 ± 2.03%) or Type 2 (“2a+X”, 7.37 ± 3.00% vs. 16.5 ± 6.03%) cell and increased neuroblasts or neuron doublets (“N+N”,16.3 ± 1.82% vs. 3.87 ± 1.65%). Cluster size (1.55 ± 0.03 vs. 1.54 ± 0.04) and overall β-Gal$^+$ cell numbers (Supplementary Fig. 5) were indistinguishable in Fc-ephrin-B2 vs. control mice. Thus, ephrin-B2 signaling increases neuronal differentiation without altering proliferation. ** P <0.05; ± s.d; Five sections (10 hemispheres) analyzed in n = 4 Anti-Fc and 5 Fc-ephrin-B2 brains; dotted vs. dashed lines mark SGZ/Hilus vs. GCL/MCL boundaries.

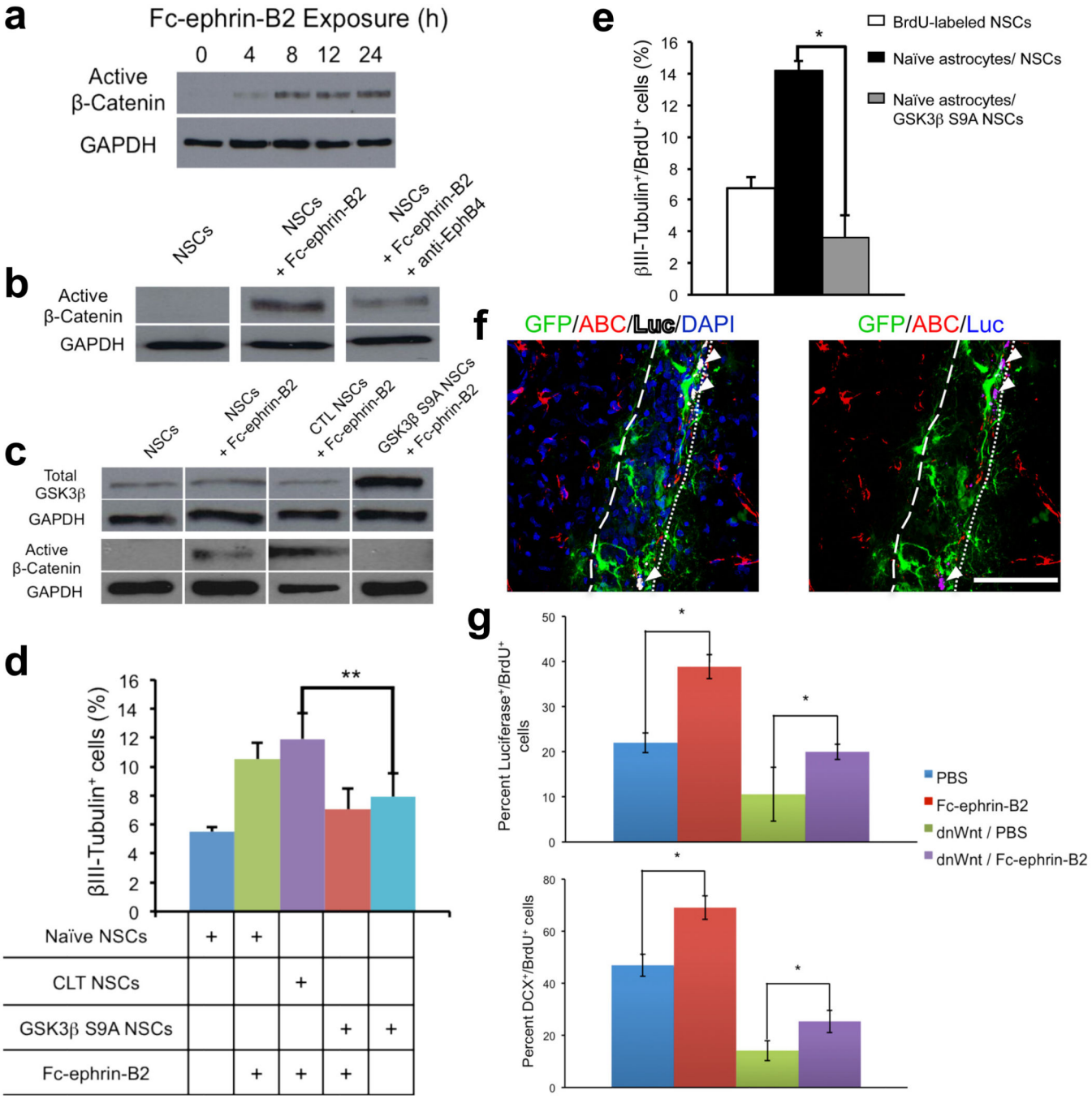

Figure 7.

Ephrin-B2 instructs neuronal differentiation by activating β-catenin independent of Wnt signaling. (**a**) Fc-ephrin-B2 (10 μg/mL) induced active β-catenin accumulation in NSCs over a 24 hours. (**b**) However, blocking the EphB4 receptor compromised Fc-ephrin-B2 induction of β-catenin accumulation. (**c**) NSCs expressing a constitutively active GSK3 β, GSK3 ββS9A, did not accumulate β-catenin in response to Fc-ephrin-B2 (10 μg/mL for 24 hours), in contrast to naïve or empty vector control NSCs (CTL NSCs). (**d**) Constitutive degradation of β-catenin in GSK3 βS9A NSCs decreased NSCs differentiation into βIII-Tubulin^{+}

neurons in response to Fc-ephrin-B2 (10 μg/mL) vs. empty vector control NPCs (CTL NPCs) (*n* = 3 experimental replicates). (**e**) The lack of β-catenin signaling in GSK3 βS9A NSCs also nullified the proneuronal effect of hippocampus-derived, ephrin-B2 expressing astrocytes in co-culture (n=4, experimental repeats). (**f**) In mice co-infected with Tcf-Luc and dnWnt-IRES-GFP constructs, cells in the SGZ still expressed active β-catenin (ABC) and Luciferase (arrowheads) 24 hours after Fc-ephrin-B2 injection. Scale bar represents 100 μm. (**g**) In hippocampi co-infected with Tcf-Luc and either dnWnt-IRES-GFP or IRES-GFP construct, then injected with Fc-ephrin-B2 or PBS, Fc-ephrin-B2 increased the percentage of SGZ $BrdU^+$ cells with active β-catenin signaling 24 hours post-injection and the percentage of $DCX^+/BrdU^+$ cells by day 5 even with dnWnt present (n=4 brains, 8 sections per brain). Ephrin-B2 thus activates β-catenin signaling and enhances adult neurogenesis independent of Wnt signaling. * indicates $P < 0.01$; ** indicates $P < 0.05$; ± s.d; dotted vs. dashed lines mark SGZ/Hilus vs. GCL/MCL boundaries. Full length blots in Supplementary Fig. 9.