Biomaterial Microenvironments to Support the Generation of New Neurons in the Adult Brain

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Key Words. Adult stem cells • Neural stem cells • Stem cell-microenvironment interactions • Neural differentiation

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ABSTRACT

Neural stem cells (NSC) in two regions of the adult mammalian brain—the subventricular zone (SVZ) and hippocampus—continuously generate new neurons, enabled by a complex repertoire of factors that precisely regulate the activation, proliferation, differentiation, and integration of the newborn cells. A growing number of studies also report low-level neurogenesis in regions of the adult brain outside these established neurogenic niches—potentially via NSC recruitment or activation of local, quiescent NSCs—under perturbations such as ischemia, cell death, or viral gene delivery of proneural growth factors. We have explored whether implantation of engineered biomaterials can stimulate neurogenesis in normally quiescent regions of the brain. Specifically, recombinant versions of factors found within the NSC microenvironment, Sonic hedgehog, and ephrin-B2 were conjugated to long polymers, thereby creating highly bioactive, multivalent ligands that begin to emulate components of the neurogenic niche. In this engineered biomaterial microenvironment, new neuron formation was observed in normally non-neurogenic regions of the brain, the striatum, and the cortex, and combining these multivalent biomaterials with stromal cell-derived factor-1α increased neuronal commitment of newly divided cells seven- to eightfold in these regions. Additionally, the decreased hippocampal neurogenesis of geriatric rodents was partially rescued toward levels of young animals. We thus demonstrate for the first time de novo neurogenesis in both the cortex and striatum of adult rodents stimulated solely by delivery of synthetic biomaterial forms of proteins naturally found within adult neurogenic niches, offering the potential to replace neurons lost in neurodegenerative disease or injury as an alternative to cell implantation. STEM CELLS 2014;32:1220–1229

INTRODUCTION

Two specific regions of the adult mammalian central nervous system (CNS) harbor active neural stem cells (NSCs): the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), a region that plays central roles in several forms of learning and memory [1], and the subventricular zone (SVZ) of the lateral ventricles, which gives rise to neurons that migrate to the olfactory bulb [2]. These neurogenic regions harbor unique cellular and biochemical microenvironments that both enable and control their neurogenic potential. For example, bone morphogenic protein [3] and notch [4, 5] signaling modulate the balance between quiescent and proliferative NSCs in the hippocampus, and Sonic hedgehog (Shh) [6], fibroblast growth factor-2 [7], vascular endothelial growth factor [8], and Wnt7a [9] regulate NSC proliferation. Additionally, ephrin-B2 [10], Wnt3a [11], and gamma aminobutyric acid (GABA) inputs from local neuronal circuitry [12] regulate neuronal differentiation.

Furthermore, neurogenesis decreases with age [13], likely due to imbalances in biochemical factors and an accompanying decline in NSC function [14–16].

In addition to broadly accepted evidence for two neurogenic regions in adult mammals, some reports suggest neurogenesis in other CNS regions under certain conditions. For example, introducing a reactive chromophore to induce apoptosis of corticothalamic neurons led to low levels of apparent new neuron formation in associated regions [17]. Focal cerebral ischemia has also been reported to induce aberrant neurogenesis in several locations—including the cortex [18, 19], striatum [20], and SVZ [21]—and the latter reportedly generated neuroblasts that migrated and subsequently differentiated into neurons appropriate to the infracted regions [21]. Furthermore, chemical ablation of dopaminergic neurons in the adult mammalian substantia nigra [22] and midbrain [23] was reportedly sufficient to induce modest proliferation and subsequent dopaminergic differentiation of endogenous quiescent neural

progenitor cells, a small number of which survive beyond 2 weeks. Finally, neurodegeneration in the hypothalamus due to mitochondrial dysfunction induced cell proliferation and generation of regionally specific neuronal subtypes as a potential compensatory mechanism [24].

While these studies raise the promising possibility that endogenous NSCs could be activated or recruited for potential cell replacement therapies, such as in the striatum for Parkinson's or Huntington's diseases or cortex for Alzheimer's disease, considerable tissue damage or inflammation is often required. Furthermore, while these studies establish that de novo neurogenesis can occur, the levels, fates, and locations of the new neurons could be further controlled and enhanced. To address these needs, recent studies have used gene delivery of pro-neurogenic factors into the rodent neocortex [25] and neostriatum [26], with and without inducing ischemia, respectively. The resulting sustained overexpression of the factors led to moderate increases in de novo neurogenesis.

Biomaterials functionalized with ligands that are present in natural neurogenic regions may offer a transient, biomedically advantageous microenvironment to stimulate neuronal regeneration within damaged or diseased regions of the brain. The finite half-life of wild-type proteins may limit the efficacy of protein delivery for stimulating the generation and maturation of new neurons. However, we have recently engineered multivalent ligands, composed of proteins conjugated to a linear biopolymer, which cluster their cognate receptors and thereby substantially elevate signaling relative to corresponding monovalent ligands [27–31]. This raises the possibility that the ligands found within the stem cell niche could be integrated into highly bioactive materials and thereby begin to create a synthetic stem cell microenvironment for controlling cell fate decisions. For example, one recent study used a functionalized material coating on the surface of bone implants to promote osseointegration, potentially by manipulating osteoprecursors in situ [31]. Additionally, we have recently established that biomaterials presenting individual bioactive factors have the ability to significantly increase the neuronal differentiation of NSCs in the adult hippocampus [28]. However, these two studies utilized single factors, and no studies have explored the potential for bioactive microenvironments to present combinations of signals to stimulate endogenous stem or progenitor cells and induce their ectopic differentiation, particularly in delicate tissues such as the CNS.

Accordingly, we have engineered soluble, multivalent biomaterial conjugates harboring factors present within the naturally neurogenic hippocampus, specifically ephrin-B2 [10] and Shh [6, 29], and these materials were injected into the interstitial space of both the striatum and cortex of the adult brain to create a synthetic, engineered stem cell microenvironment that stimulates the proliferation and neuronal differentiation of normally quiescent progenitor cells. Additionally, by including the chemokine stromal cell-derived factor-1α (SDF-1α), a more than fourfold increase in the basal level of neurogenesis compared with controls was observed, suggesting potential recruitment of endogenous NSCs from the neurogenic SGZ and SVZ to the normally non-neurogenic regions. Therefore, synthetic stem cell microenvironments represent a new strategy to induce de novo neurogenesis and thereby potentially replace neurons lost to neurodegenerative disease or traumatic brain injury.

MATERIALS AND METHODS

Recombinant Protein Production, Purification, and Multivalent Biomaterial Conjugation

Murine ephrin-B2 ectodomain sequence (amino acids 31–227) was amplified from the plasmid pcDNA3.1-*ephrin-B2*-hFc, and rat Shh N-terminal signaling domain (amino acids 25–198) was produced as described previously [6]. A C-terminal hexahistidine tag and cysteine were added during polymerase chain reaction, followed by insertion into the bacterial expression plasmid pBAD. Protein was expressed and purified as described previously [30]. Protein purity was assessed by confirmation of a single band following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified ephrin-B2 was conjugated to 800 kDa hyaluronic acid (HA) (Genzyme, Cambridge, MA, www.genzyme.com) through a two-step reaction using carbodiimide chemistry at the HA carboxylate group and a maleimide reaction at the protein C-terminal cysteine [30]. In the first step, 3,3′-*N*-(ε-maleimidocaproic acid) hydrazide (EMCH, Pierce, Rockford, IL, www.piercenet.com, 1.2 mg/ml), *N*-hydroxysulfosuccinimide (Sulfo-NHS, Pierce, Rockford, IL, www.piercenet.com, 2.8 mg/ml), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce, Rockford, IL, www.piercenet.com, 10 mg/ml) were added to a 3-mg/ml solution of HA in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Sigma, St. Louis, MO, www.sigmaaldrich.com) buffer pH 6.5 and allowed to react at 4°C for 4 hours. The solution was then dialyzed into pH 7.0 phosphate buffered saline containing 10% glycerol and 2 mM EDTA. Recombinant ephrin-B2 was reduced using 200-fold molar excess Tris(2-carboxyethyl)phosphine hydrochloride (Pierce, Rockford, IL, www.piercenet.com) at 4°C for 5 minutes. Activated HA-EMCH was then added at desired molar ratios with reduced ephrin-B2 and allowed to react at 4°C overnight. To remove unreacted ephrin from ephrin-conjugated HA, the solution was then dialyzed with 100 kDa MWCO tubing (Spectrum Labs, Rancho Dominguez, CA, www.spectrumlabs.com) in pH 7.0 phosphate buffered saline with 2 mM EDTA. Purified ephrin and HA-conjugated ephrin protein concentrations were measured using a bicinchoninic acid assay, and valencies were verified using size-exclusion chromatography coupled with multi-angle light scattering as described previously [30].

Antibody-Clustered Ephrin-B2 Formation

To create clustered ephrin-B2 complexes (Fc-ephrin-B2), recombinant mouse ephrin-B2/Fc chimera (R&D Systems, Minneapolis, MN, www.rndsystems.com) was incubated with goat anti-human IgG, Fc-fragment specific (Jackson ImmunoResearch, West Grove, PA, www.jacksonimmuno.com) antibody at a 1:9 ratio (w/w), which led to maximal activity of the resulting clusters (data not shown). After 90 minute at 4°C, complexes were immediately used.

Stereotactic Injections

All animal protocols were approved by the University of California, Berkeley Animal Care and Use Committee and conducted in accordance with National Institutes of Health guidelines. An 8-week-old adult female rat and a 19-month-old adult male Fisher 344 rat were intraperitoneally injected with halogenated thymidine analog (163 μmol/kg) for 3 days

before surgery. On the fourth day, each animal was anesthetized with a ketamine/xylazine cocktail and underwent bilateral intrahippocampal stereotactic injections with 3 μl of either HA (4.7 nM) or multivalent protein (4.7 nM HA and 282 nM protein). The hippocampal injection coordinates with respect to bregma were 3.5 mm anteriorposterior (AP), −3.3 mm dorsoventral (DV) (from dura), and ±1.8 mm mediolateral (ML). Striatal coordinates used were 0.3 mm AP, −5.0 mm DV, and ±3.5 mm ML. Cortical coordinates used were 0.3 mm AP, −0.7 mm DV, and ±3.5 mm ML. For the study involving SDF-1α, striatal coordinates were −1.0 mm AP, −4.0 mm DV, and ±3.0 mm ML, and cortical coordinates were −1.0 mm AP, −1.0 mm DV, and ±3.0 mm ML. To assess neuronal maturation, two subsequent stereotactic injections were performed 30 days apart. For cortical and striatal injections, an Alexa 488-dextran conjugate (Molecular Probes, cat. #D-22910, Carlsbad, CA, www.lifetechnologies.com) was included in the injection solution (20 mg/ml final concentration in phosphate buffered saline) to label the injection region for quantification purposes. Before injections, animals received a buprenorphine/meloxicam cocktail in saline as an analgesic and to avoid dehydration. Directly after the surgery a 1 ml intraperitoneal injection of yohimbine in saline was administered to counteract the effects of xylazine. Another injection of buprenorphine/meloxicam in saline was provided 6–8 hours and 24 hours postsurgery as additional analgesic. On the sixth day after surgery, the rats were perfused with 4% PFA, and brains were extracted, stored in fixative for 24 hours, and allowed to settle in a 30% sucrose solution before processing for immunostaining.

Immunostaining

Coronal brain sections (40 μm) were processed, stored, and stained as described previously [32]. Primary antibodies used were mouse antibody to BrdU (1:100, Roche, cat. #11170376001, Basel, Switzerland, www.roche.com), mouse antibody to NeuN (1:100, Millipore, cat. #MAB377, Billerica, MA, www.millipore.com), mouse antibody to nestin (1:1,000, BD Pharmingen, cat. #556309, San Jose, CA, www.bdbiosciences.com), guinea pig antibody to doublecortin (1:1,000, Millipore, cat. #AB2253, Billerica, MA, www.millipore.com), goat antibody to ephrin-B2 (1:10, R&D Systems, cat. #AF496, Minneapolis, MN, www.rndsystems.com), rabbit antibody to Sox2 (1:250, Millipore, cat. #AB5603, Billerica, MA, www.millipore.com), goat antibody to EphB4 (1:50, Santa Cruz, cat. #sc-7285, Santa Cruz, CA, www.scbt.com), rat antibody to CldU (1:500, Novus Biologicals, cat. #NB500-169, Littleton, CO, www.novusbio.com), and mouse antibody to IdU (1:25, Becton Dickinson, cat. #347580, Franklin Lakes, NJ, www.bd.com). Appropriate Cy3-, Cy5- or Alexa Fluor 488–conjugated secondary antibodies (1:125, Jackson Immunoresearch, West Grove, PA, www.jacksonimmuno.com; affinity-purified whole IgG with minimal cross-reactivity, 1:250, Life Technologies, Carlsbad, CA, www.lifetechnologies.com, Alexa Fluor-conjugated secondary antibodies) were used. For sections stained with rat antibody to CldU, biotin-conjugated antibody to rat IgG (1:250, Jackson Immunoresearch, cat. #712-065-150, West Grove, PA, www.jacksonimmuno.com) was used as the secondary, which was then washed and incubated with Cy3-conjugated streptavidin (1:1,000, Jackson Immunoresearch, cat. #016–160-084, West Grove, PA, www.jacksonimmuno.com) for 2 h to amplify the signal. DAPI (20 μg/ml, Invitrogen, Carlsbad, CA, www.lifetechnologies.com) was used as a nuclear counterstain.

Sections were then mounted on glass slides, and either stereological analysis (Stereo Investigator, MBF Biosciences, Williston, VT, www.mbfbioscience.com) or confocal microscopy (Zeiss LSM 710, Jena, Germany, www.zeiss.com) was performed. In short, using an optical fractionator method, marker+ cells were counted in a Systematic Randomly Sampled (SRS) set of unbiased virtual volumes inside the SGZ and granular cell layer of both the left and right sides of the hippocampus. For striatal and cortical sections, marker+ cells were quantified inside bulk volumes positive for dextran-488. An estimate of the total number of marker+ cells in brain regions was then generated.

Statistical Analysis

Statistical significance of the results was determined using an ANOVA and multiple means comparison function (Tukey-Kramer method) in MATLAB with an alpha level of 0.05 unless otherwise noted. All error bars are reported in SD from the mean, with $n = 3$ unless otherwise noted.

RESULTS

EphB4[+] Cells that Also Express Neural Progenitor Markers Exist Throughout the Brain

We recently discovered that the transmembrane protein ligand ephrin-B2 is a strong regulator of adult hippocampal neurogenesis. In particular, ephrin-B2 expressed from hippocampal astrocytes activates the EphB4 receptor on neighboring NSCs to induce neuronal fate commitment in the hippocampal SGZ [10]. To examine the broader expression of ephrin-B2 and its receptor EphB4, we performed immunostaining of the hippocampus (Fig. 1A) and various non-neurogenic regions of the brain, including the striatum (Fig. 1B) and cortex (Fig. 1C). Interestingly, cells positive for the receptor EphB4 were present throughout the brain, a subset of which coexpressed the NSC marker Sox2 in the SGZ, cortex, and striatum. The neurogenic ligand ephrin-B2, however, was markedly absent or spatially separate from EphB4^{+} cells in the normally non-neurogenic striatum and cortex, respectively. Due to the juxtacrine nature of Eph-ephrin interactions, the absence of ligand-expressing cells in these regions would indicate a lack of EphB4 signaling, which could potentially contribute to the absence of neurogenesis under normal physiological conditions. Furthermore, previous studies have reported a notable absence of Shh expression in both the striatum and the upper layers of the cortex, whereas its receptor Patched and associated protein Smoothened were often present [33].

Highly Multivalent Ephrin-B2 Enhances Hippocampal Neurogenesis and Induces De Novo Striatal and Cortical Neurogenesis

Ectopic activation of Eph/ephrin signaling—which naturally requires the assembly or clustering of receptor-ligand complexes [34]—currently involves the addition of antibody-clustered ephrin ectodomains, a method that requires high protein concentrations and involves poorly controlled clustering of Eph receptors. We have recently shown that creating multivalent biomaterials, composed of multiple ligands conjugated to a linear polymer, can cluster receptors and dramatically increase the bioactivity of a given protein [28, 30].
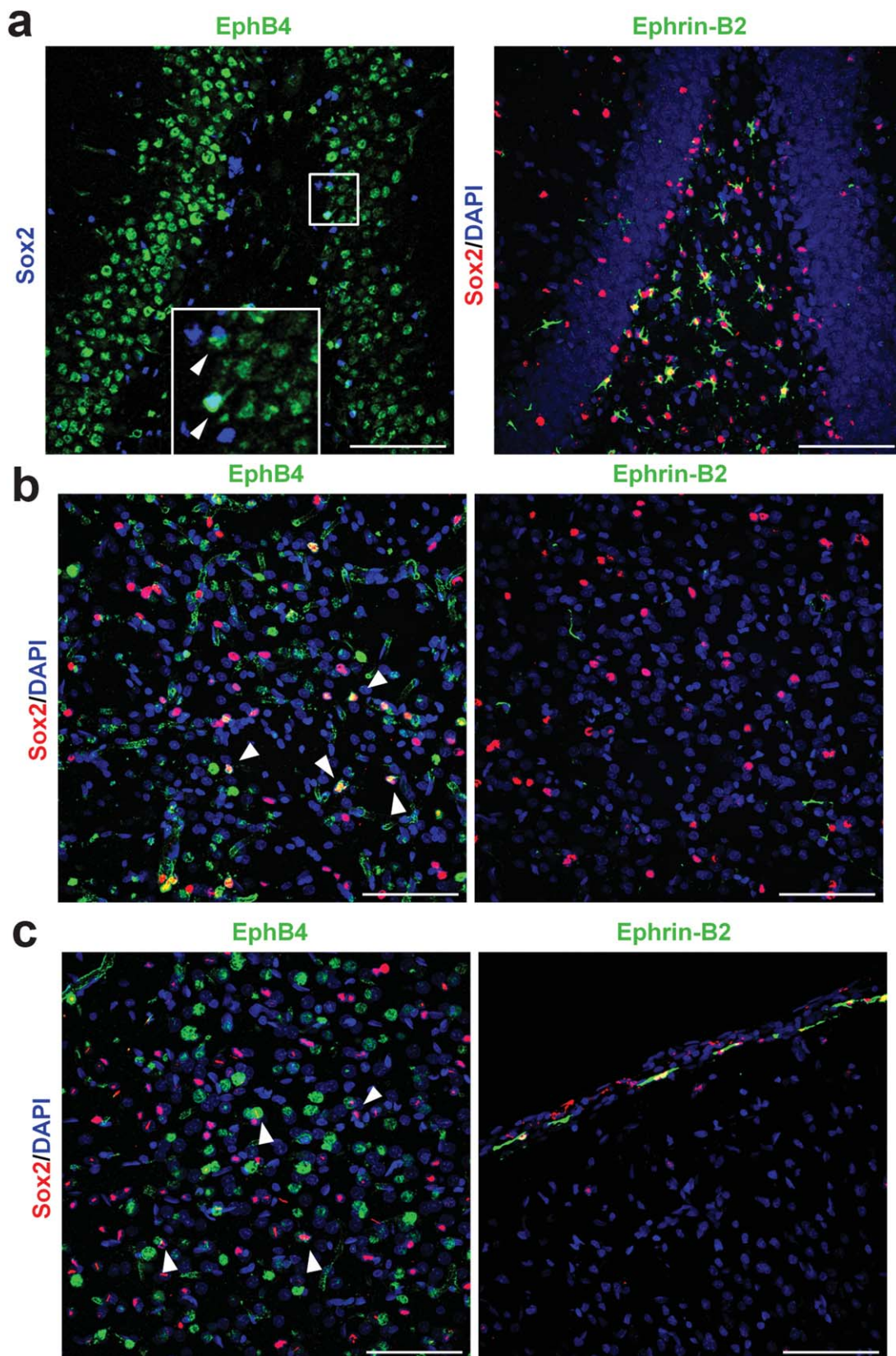
Figure 1. Expression of EphB4 and ephrin-B2 in the adult brain. (A): Representative image of the adult hippocampus containing EphB4^{+} cells, a subset of which are positive for the neural stem cell (NSC) marker Sox2 (high magnification inset), in close proximity to its neurogenic ligand ephrin-B2 (right panel). (B, C): Representative image of the adult striatum (B) and cortex (C), containing EphB4^{+} cells expressing the NSC marker Sox2 but devoid of the neurogenic ephrin-B2 signal. Scale bar = 100 μm. Arrowheads point to relevant cells. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
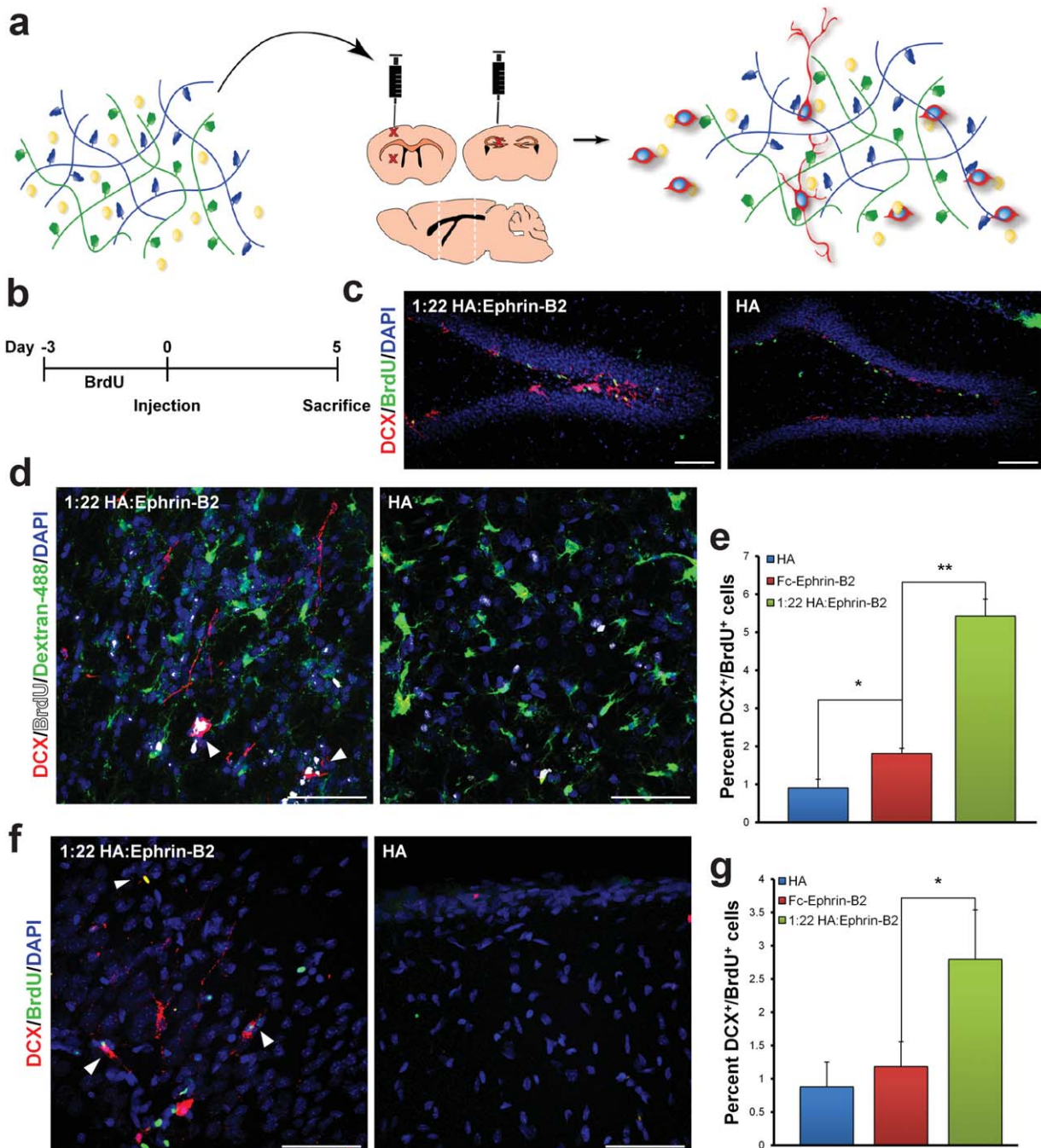
Figure 2. Highly multivalent ephrin-B2 enhances hippocampal neurogenesis and induces de novo striatal and cortical neurogenesis. (A): Schematic representation of combinatorial multivalent biomaterial (blue and green) and soluble chemokine (yellow) injection into the brain to attract neural stem cells (red) and subsequently differentiate them into neurons. (B): Schematic representation of experimental timeline. (C): Representative image of increased hippocampal neurogenesis resulting from highly multivalent ephrin-B2 administration versus hyaluronic acid (HA) control. (D): Representative image of de novo striatal neurogenesis and HA control. The cell marker dextran-488 labels the site of stereotactic injection. (E): Quantification of fraction of newly divided cells expressing the early neuronal marker DCX after striatal stereotactic injection. (F): Representative image of de novo cortical neurogenesis and HA control. (G): Quantification of fraction of newly divided cells expressing the early neuronal marker DCX after cortical injection. Scale bar = 100 μm. Arrowheads point to relevant cells. *, $p < .05$; **, $p < .01$. Error bars represent $\pm$SD. Abbreviations: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; HA, hyaluronic acid.

Combining several such multivalent ligands along with soluble chemokines may begin to reconstruct the bioactive NSC niche (Fig. 2A). After 3 days of bromodeoxyuridine injection to mark mitotic cells, we injected a multivalent form of ephrin-B2 to determine the ability of exogenous ephrin-B2 administration

to enhance neurogenesis in the hippocampus after 5 days (Fig. 2B). Antibody-clustered ephrin-B2 (Fc-ephrin-B2) administration significantly increased the fraction of mitotically labeled cells that acquired markers indicative of neuronal fate commitment $(60.1 \pm 2.14\%)$ compared with HA controls

$(49.9 \pm 3.61\%)$ $(p < .05)$, which in general were similar in counts to uninjected brains [28]. Furthermore, consistent with our prior results [28], highly multivalent ephrin-B2 (1:22 HA:ephrin-B2) substantially enhanced the extent of neuronal differentiation in this region, at a threefold higher potency than Fc-ephrin-B2 $(83.9 \pm 4.92\%)$ (Fig. 2C).

As exogenous administration of highly multivalent ephrin-B2 was capable of enhancing neurogenesis in the naturally neurogenic hippocampus, we investigated whether this bioactive ligand could induce de novo neurogenesis in quiescent regions of the brain that harbor cells coexpressing its receptor EphB4 and the NSC marker Sox2. After the same experimental paradigm (Fig. 2B) and additionally injecting fluorescently labeled dextran (Dextran-488) to mark the injection site, we found that multivalent ephrin-B2 induced a sixfold increase in the fraction of newly divided cells expressing the early neuronal marker DCX in the adult rat striatum $(p < .01)$ (Fig. 2D, 2E). Interestingly, similar results were seen in the cortex, with highly multivalent ephrin-B2 inducing an almost threefold increase in early markers of neurogenesis compared with the antibody-clustered form $(p < .05)$ (Fig. 2F, 2G). These data represent the first example of de novo neurogenesis in the adult rodent brain resulting from solely recombinant protein or biomaterial administration under normal physiological conditions.

Dual Administration of Multivalent Conjugates Enhances Short- and Long-Term Hippocampal Neurogenesis and De Novo Striatal and Cortical Neurogenesis in Young Rats

Adult neurogenic niches present factors that regulate both proliferation and differentiation; therefore, to take an additional step toward an ectopic neurogenic environment, Sonic hedgehog (Shh) was utilized in addition to bioactive ephrin-B2. To track both long- and short-term neurogenesis, the same animal, two separate systemic administrations of two distinct thymidine analogs—chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU) —were performed 33 days apart, each followed by intracranial stereotactic injections of neurogenic factors (Fig. 3A). To test short-term bioactivity in the neurogenic SGZ, CldU was administered, and multivalent biomaterial forms of both ephrin-B2 (1:22 HA:ephrin-B2) and Shh (1:16 HA:Shh) were injected into the hippocampus either separately or combined (ephrin-B2/Shh) (Fig. 3B). Subsequently, the overall number of newly divided CldU$^+$ cells (Fig. 3C) and the fraction of new neurons (Fig. 3D) were quantified. While multivalent Shh administration—either singly or in combination with multivalent ephrin-B2—did not statistically increase the number of newly divided cells 5 days after injection, groups containing highly multivalent ephrin-B2 showed significantly higher levels of hippocampal neurogenesis compared with controls $(p < .05)$. The differential specificity of the antibody used to detect IdU, as well as the difference in propensity of IdU to integrate into the host DNA of dividing NSCs [35] may explain the modest difference in the fraction of colabeled DCX$^+$ cells compared with previous experiments using BrdU (Fig. 2C).

We next assessed whether Shh and ephrin-B2 administration to a non-neurogenic region could modulate short-term proliferation or differentiation. Furthermore, several studies have used chemokines to attract stem cells surrounding an implanted material to promote tissue integration—primarily in the bone [36, 37], muscle [38], and cartilage [39, 40]—and

SDF-1α has been reported to modulate NSC migration in vivo [41, 42]. Therefore, we also included this molecule as a soluble factor to potentially recruit nearby, active NSCs from the SVZ and thereby take steps toward a more complete synthetic stem cell microenvironment. In this case, highly multivalent Shh in conjunction with ephrin-B2 showed a significant proliferative effect compared with controls $(p < .05)$ (Fig. 3E, 3F). Furthermore, the administration of the chemokine SDF-1α together with ephrin-B2 and Shh biomaterials (ephrin-B2/Shh/SDF-1) induced a sevenfold increase in the basal level of neurogenesis in the region compared with vehicle control (Fig. 3G), suggesting that a combinatorial effect of de novo neurogenesis and possible recruitment of active NSCs followed by signaling from neurogenic factors potentially occurred. The combination of ephrin-B2 and Shh was also administered into the cortex (Fig. 3H) and yielded a significant increase in the number of newly divided cells (Fig. 3I) and a nearly eightfold increase fraction of neurons formed over a 5-day period postintracranial injection relative to the vehicle control $(p < .05)$ (Fig. 3J).

The introduction of factors from an active neurogenic stem cell niche thus induced early stages of neurogenesis in normally quiescent regions of the brain, raising the question of whether these newborn neurons could undergo long-term survival. Therefore, the fate of cells that had divided and were therefore labeled by IdU between 38 and 41 days previously and were exposed to neurogenic factors 38 days previously was analyzed. In the hippocampus (Fig. 4A), highly multivalent Shh significantly increased the number of IdU$^+$ cells $(p < .05)$ (Fig. 4B). Furthermore, exposure to highly multivalent ephrin-B2 increased the fraction of such IdU$^+$ cells expressing the mature neuronal marker NeuN 38 days later (Fig. 4C).

Within the ordinarily non-neurogenic striatum (Fig. 4D), Shh did not increase the number of IdU-labeled cells observed after 38 days (Fig. 4E); however, the fraction of IdU+ cells that differentiated into mature neurons was over twofold greater with ephrin-B2/Shh and SDF-1α administration $(p < .05)$ (Fig. 4F). A similar trend was observed in the cortex (Fig. 4G, 4H, 4I), suggesting that a significant fraction of new neurons born in normally quiescent regions ($\sim$7–8% of dividing cells) survived for long periods of time ($\sim$2.5% of dividing cells) posttreatment in the presence of ephrin-B2/Shh/SDF-1α.

Multivalent Conjugates Partially Rescue Short-Term Hippocampal Neurogenesis in Geriatric Rats

Finally, it has been well-documented [13, 14, 16] that there is a substantial decline in adult hippocampal neurogenesis with organismal aging; therefore, we explored whether administration of highly bioactive, synthetic forms of factors naturally present within the young neurogenic stem cell niche could partially restore this deficit. Using a similar experimental timeline as the previous study (Fig. 5A), we injected highly multivalent Shh and ephrin-B2 separately or in combination. After 38 days postinjection, the combination showed no significant change $(P < 0.402)$ in the number of newly divided cells or fraction of mature neurons remaining compared with controls (Fig. 5B, 5C, 5D). However, the combination of bioactive materials (Fig. 5E) was found to significantly increase the number of newly divided cells compared with controls $(p < .075)$ (Fig. 5F), while highly multivalent ephrin-B2 was capable of increasing the fraction of labeled cells that underwent
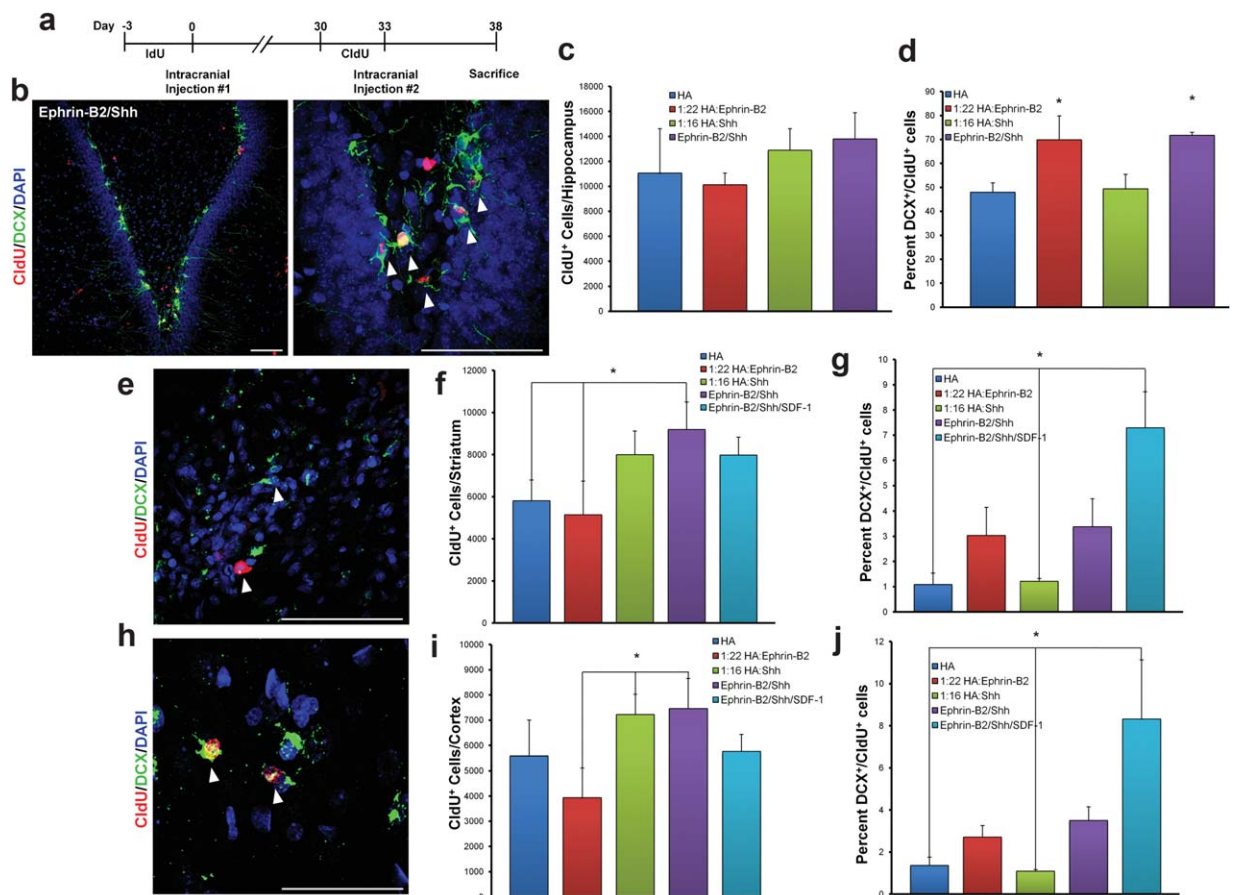
Figure 3. Dual administration of ephrin-B2 and Shh multivalent conjugates enhances short-term hippocampal neurogenesis and de novo striatal and cortical neurogenesis. (A): Schematic representation of experimental timeline. (B): Representative image of increased hippocampal neurogenesis resulting from coadministration of multivalent ephrin-B2 and Shh (ephrin-B2/Shh). (C): Quantification of total number of newly divided cells × days after hippocampal stereotactic injection. (D): Quantification of fraction of newly divided cells expressing the early neuronal marker DCX 5 days after hippocampal stereotactic injection. (E): Representative image of de novo striatal neurogenesis. (F): Quantification of total number of newly divided cells after striatal stereotactic injection. (G): Fractions of newly divided cells expressing the early neuronal marker DCX after striatal stereotactic injection. (H): Representative image of de novo cortical neurogenesis. (I): Quantification of total number of newly divided cells after stereotactic cortical injection. (J): Fractions of newly divided cells expressing the early neuronal marker DCX after stereotactic cortical injection. Scale bar = 100 μm. Arrowheads point to relevant cells. *, $p < .05$. Error bars represent ±SD. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HA, hyaluronic acid.

neuronal differentiation within 5 days (Fig. 5G) to levels comparable to that of young animals [28]. Additional molecular engineering of the bioactive material, such as designing strategies for controlled release or chemical stabilization of the polymer backbone, may promote sustained signaling that may be able to fully rescue the level of neurogenesis in elderly individuals to that of young ones.

DISCUSSION

We have explored the concept of whether biomaterials can be engineered to create an ectopic, in vivo stem cell microenvironment, and these results demonstrate for the first time de novo neurogenesis in the adult brain under physiologically normal conditions using only recombinant proteins, as components of highly bioactive materials. In addition, this work explored three signaling molecules found within the neurogenic stem cell niche—ephrin-B2, Shh, and SDF-1α—

and the platform can be further engineered to modulate additional factors such as the notch signaling pathway [5], Noggin and BMP receptors [3], Wnt3a [11], and the neurotransmitter GABA [43]. Interestingly, the endogenous cellular sources of the factors used in this study varied from astrocytes within the hilus [10], neurons of the ventral forebrain that project axons into the DG [44], to interneurons, and to endothelial cells within the DG [45]. Receptors for all these factors, however, are expressed by neural stem and progenitor cells of the SGZ as well as immature and mature neurons of the granular cell layer [6, 10, 45]. A combinatorial approach to deliver these factors as well as additional factors from the neurogenic stem cell niche into damaged or degenerated regions of the brain may further override endogenous, non-neurogenic microenvironments to activate quiescent NSCs, or recruit nearby NSCs and form new neurons. Furthermore, despite NSCs having been successfully cultured from non-neurogenic regions of the brain, it is still unclear whether these cells exist as quiescent endogenous progenitors
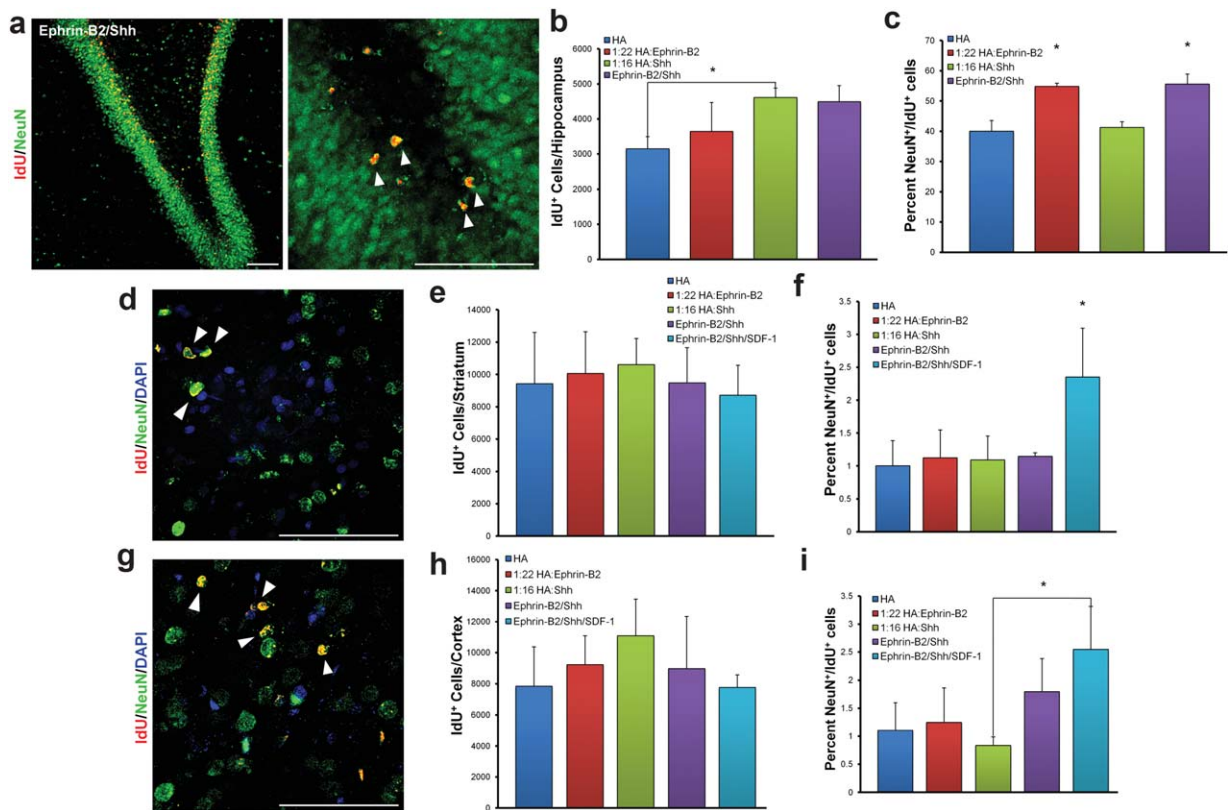
Figure 4. Dual administration of multivalent conjugates enhances long-term hippocampal neurogenesis and de novo striatal and cortical neurogenesis. (A): Representative image of increased hippocampal neurogenesis resulting from dual multivalent ephrin-B2 and Shh administration (ephrin-B2/Shh). (B): Quantification of total number of newly divided cells after hippocampal stereotactic injection. (C): Fractions of newly divided cells expressing the mature neuronal marker NeuN 38 days after intracranial injection of biomaterials. (D): Representative image of de novo striatal neurogenesis. (E): Quantification of total number of newly divided cells after striatal stereotactic injection. (F): Fractions of newly divided cells expressing the mature neuronal marker NeuN after striatal injection. (G): Representative image of de novo cortical neurogenesis. (H): Quantification of total number of newly divided cells after cortical stereotactic injection. (I): Fractions of newly divided cells expressing the mature neuronal marker NeuN after cortical stereotactic injection. Scale bar = 100 μm. Arrowheads point to relevant cells. *, $p < .05$. Error bars represent ±SD. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HA, hyaluronic acid.
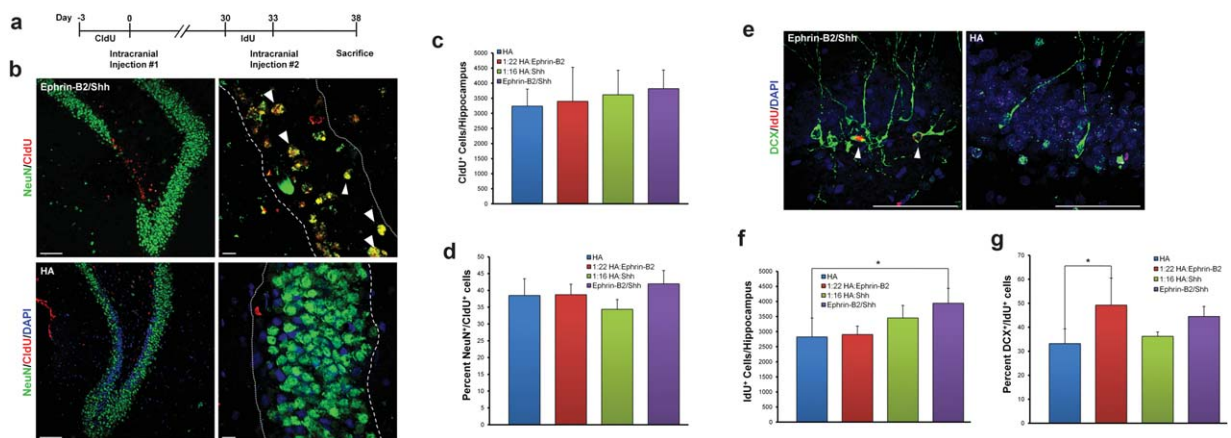

Figure 5. Multivalent conjugates partially rescue short-term hippocampal neurogenesis in male geriatric rats. (A): Schematic representation of experimental timeline. (B): Representative image of hippocampus after dual multivalent ephrin-B2 and Shh (ephrin-B2/Shh) administration versus hyaluronic acid (HA) control. (C): Quantification of total number of newly divided cells 38 days after hippocampal injection of biomaterials. (D): Quantification of fraction of newly divided cells expressing the mature neuronal marker NeuN 38 days after hippocampal injection of biomaterials. (E): Representative image of increased hippocampal neurogenesis resulting from dual multivalent ephrin-B2 and Shh administration versus HA control. (F): Quantification of total number of newly divided cells 5 days after hippocampal injection. (G): Fractions of newly divided cells expressing the early neuronal marker DCX 5 days after hippocampal injection. Scale bar = 100 μm. Arrowheads point to relevant cells. *, $p < .08$. Error bars represent ±SD. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; HA, hyaluronic acid.

or are reprogrammed due to cell culture conditions that often include serum. This work provides evidence supporting the existence of quiescent progenitors because even in the absence of the NSC migration factor SDF-1α, local administration of pro-neurogenic factors immobilized to high-molecular-weight soluble biomaterials are capable of inducing a three- to fivefold increase in neuronal differentiation in normally inactive regions of the brain compared with controls (Figs. 2E, 2G, 3G, 3J). Moreover, the marker EphB4 could also potentially serve as a marker to partially identify endogenous stem and progenitor cells (Fig. 1). Future work may explore the extent to which these cells arise from local quiescent cells or are recruited from active neurogenic niches.

Several hurdles must be overcome for this approach to represent a viable therapeutic strategy. First, it is not sufficient to produce new neurons, but those cells must also be of the appropriate neuronal subtype and in most cases must appropriately integrate into the existing neuronal circuitry [46]. For example, many of the neurons that would be therapeutically attractive to generate in the adult CNS were originally created long ago during organismal development, and it will thus be challenging to recapitulate the relevant neurodevelopmental niches to generate the appropriate neuronal subtype within adults. A more thorough, basic understanding of stem cell self-renewal and differentiation mechanisms will further enable cell replacement therapy efforts, potentially aided by synthetic microenvironments presenting additional cues [47, 48]. Secondly, a single administration of these bioactive factors may not be sufficient to yield the numbers of cells necessary to replace the ones lost due to disease, which often involves progressive neuronal degeneration. Therefore, a long-term biomaterial delivery system, with appropriate release kinetics [49] and tuning of relative cell self-renewal and differentiation, may enable levels of neurogenesis and long-term neuronal survival necessary to repair affected regions. Finally, correct functional integration of newborn neurons must be achieved for neurological recovery. Functionalized biomaterials containing axonal migration factors could conceivably be used to promote long-distance axonal projection of newly differentiated neurons into their appropriate regional target in the brain [50].

SUMMARY

In summary, we propose a novel approach for engineering synthetic materials to promote newborn neurons in regions of the brain that are predominantly quiescent. Our findings raise the possibility of utilizing a blend of biomaterials and bioactive factors to manipulate stem cell fate in other tissues. Future work to encompass additional factors from stem cell niches, as well characterize the fate and function of newborn neurons, will take steps closer to potential clinical applications.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

A.C.: performed all of the experiments and analyzed all data; A.C. and D.V.S.: designed the experiments and wrote the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

D.V.S. is an inventor on a patent involving polyvalent ligands as potent activators of cellular signaling. A.C. indicate no potential conflicts of interest.

REFERENCES

1 Clelland CD, Choi M, Romberg C et al. A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 2009;325:210–213.

2 Lois C, Alvarez-Buylla A. Long-distance neuronal migration in the adult mammalian brain. Science 1994;264:1145–1148.

3 Mira H, Andreu Z, Suh H et al. Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. Cell Stem Cell 2010;7:78–89.

4 Ehm O, Goritz C, Covic M et al. RBPJkappa-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus. J Neurosci 2010;30:13794–13807.

5 Lugert S, Basak O, Knuckles P et al. Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. Cell Stem Cell 2010;6:445–456.

6 Lai K, Kaspar BK, Gage FH et al. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 2003;6:21–27.

7 Jin K, Sun Y, Xie L et al. Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. Aging Cell 2003;2:175–183.

8 Cao L, Jiao X, Zuzga DS et al. VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet 2004;36:827–835.

9 Qu Q, Sun G, Li W et al. Orphan nuclear receptor TLX activates Wnt/beta-catenin signalling to stimulate neural stem cell proliferation and self-renewal. Nat Cell Biol 2010;12:31–40, 31–39.

10 Ashton RS, Conway A, Pangarkar C et al. Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling. Nat Neurosci 2012;15:1399–1406.

11 Lie DC, Colamarino SA, Song HJ et al. Wnt signalling regulates adult hippocampal neurogenesis. Nature 2005;437:1370–1375.

12 Tozuka Y, Fukuda S, Namba T et al. GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 2005;47:803–815.

13 Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J Neurosci 1996;16:2027–2033.

14 Villeda SA, Luo J, Mosher KI et al. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature 2011;477:90–94.

15 Okamoto M, Inoue K, Iwamura H et al. Reduction in paracrine Wnt3 factors during aging causes impaired adult neurogenesis. FASEB J 2011;25:3570–3582.

16 Miranda CJ, Braun L, Jiang Y et al. Aging brain microenvironment decreases hippocampal neurogenesis through Wnt-mediated survivin signaling. Aging Cell 2012;11:542–552.

17 Magavi SS, Leavitt BR, Macklis JD. Induction of neurogenesis in the neocortex of adult mice. Nature 2000;405:951–955.

18 Jiang W, Gu W, Brannstrom T et al. Cortical neurogenesis in adult rats after transient middle cerebral artery occlusion. Stroke 2001;32:1201–1207.

19 Ohira K, Furuta T, Hioki H et al. Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. Nat Neurosci 2010;13:173–179.

20 Parent JM, Vexler ZS, Gong C et al. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann Neurol 2002;52:802–813.

21 Arvidsson A, Collin T, Kirik D et al. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 2002;8:963–970.

22 Shan X, Chi L, Bishop M et al. Enhanced de novo neurogenesis and dopaminergic neurogenesis in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease-like mice. Stem Cells 2006;24:1280–1287.

23 Berg DA, Kirkham M, Beljajeva A et al. Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. Development 2010;137:4127–4134.

24 Pierce AA, Xu AW. De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. J Neurosci 2010;30:723–730.

25 Iwai M, Abe K, Kitagawa H et al. Gene therapy with adenovirus-mediated glial cell line-derived neurotrophic factor and neural stem cells activation after ischemic brain injury. Hum Cell 2001;14:27–38.

26 Benraiss A, Bruel-Jungerman E, Lu G et al. Sustained induction of neuronal addition to the adult rat neostriatum by AAV4-delivered noggin and BDNF. Gene Ther 2012;19:582.

27 Conway A, Spelke DP, Schaffer DV. Conjugation of proteins to polymer chains to create multivalent molecules. Meth Mol Biol 10.1007/7651_2013_32.

28 Conway A, Vazin T, Spelke DP et al. Multivalent ligands control stem cell behaviour in vitro and in vivo. Nat Nanotechnol 2013;8:831–838.

29 Vazin T, Ashton RS, Conway A et al. The effect of multivalent sonic hedgehog on differentiation of human embryonic stem cells into dopaminergic and GABAergic neurons. Biomaterials 2014;35:941–948.

30 Wall ST, Saha K, Ashton RS et al. Multivalency of sonic hedgehog conjugated to linear polymer chains modulates protein potency. Bioconjug Chem 2008;19:806–812.

31 Petrie TA, Raynor JE, Dumbauld DW et al. Multivalent integrin-specific ligands enhance tissue healing and biomaterial integration. Sci Transl Med 2010;2:45–60.

32 Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. Nature 1997;386:493–495.

33 Traiffort E, Charytoniuk D, Watroba L et al. Discrete localizations of hedgehog signalling components in the developing and adult rat nervous system. Eur J Neurosci 1999;11:3199–3214.

34 Davis S, Gale NW, Aldrich TH et al. Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. Science 1994;266:816–819.

35 Leuner B, Glasper ER, Gould E. Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol 2009;517:123–133.

36 Huang Q, Goh JC, Hutmacher DW et al. In vivo mesenchymal cell recruitment by a scaffold loaded with transforming growth factor beta1 and the potential for in situ chondrogenesis. Tissue Eng 2002;8:469–482.

37 Thevenot PT, Nair AM, Shen J et al. The effect of incorporation of SDF-1alpha into PLGA scaffolds on stem cell recruitment and the inflammatory response. Biomaterials 2010;31:3997–4008.

38 Fong EL, Chan CK, Goodman SB. Stem cell homing in musculoskeletal injury. Biomaterials 2011;32:395–409.

39 Erggelet C, Neumann K, Endres M et al. Regeneration of ovine articular cartilage defects by cell-free polymer-based implants. Biomaterials 2007;28:5570–5580.

40 Lee CH, Cook JL, Mendelson A et al. Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study. Lancet 2010;376:440–448.

41 Itoh T, Satou T, Ishida H et al. The relationship between SDF-1alpha/CXCR4 and neural stem cells appearing in damaged area after traumatic brain injury in rats. Neurol Res 2009;31:90–102.

42 Carbajal KS, Schaumburg C, Strieter R et al. Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. Proc Natl Acad Sci USA 2010;107:11068–11073.

43 Song J, Zhong C, Bonaguidi MA et al. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 2012;489:150–154.

44 Ihrie RA, Shah JK, Harwell CC et al. Persistent sonic hedgehog signaling in adult brain determines neural stem cell positional identity. Neuron 2011;71:250–262.

45 Bhattacharyya BJ, Banisadr G, Jung H et al. The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. J Neurosci 2008;28:6720–6730.

46 Politis M, Wu K, Loane C et al. Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. Sci Transl Med 2010;2:38–46.

47 Conway A, Schaffer DV. Biophysical regulation of stem cell behavior within the niche. Stem Cell Res Ther 2012;3:50.

48 Keung AJ, Kumar S, Schaffer DV. Presentation counts: microenvironmental regulation of stem cells by biophysical and material cues. Annu Rev Cell Dev Biol 2010;26:533–556.

49 Pritchard CD, O'Shea TM, Siegwart DJ et al. An injectable thiol-acrylate poly(ethylene glycol) hydrogel for sustained release of methylprednisolone sodium succinate. Biomaterials 2011;32:587–597.

50 Gros T, Sakamoto JS, Blesch A et al. Regeneration of long-tract axons through sites of spinal cord injury using templated agarose scaffolds. Biomaterials 2010;31:6719–6729.