Neural progenitor cells regulate microglia functions and activity

Kira I Mosher1,2,6, Robert H Andres3,5,6, Takeshi Fukuhara1,5,6, Gregor Bieri1,2, Maiko Hasegawa-Moriyama1,5, Yingbo He1, Raphael Guzman3,5 & Tony Wyss-Coray1,4

We found mouse neural progenitor cells (NPCs) to have a secretory protein profile distinct from other brain cells and to modulate microglial activation, proliferation and phagocytosis. NPC-derived vascular endothelial growth factor was necessary and sufficient to exert at least some of these effects in mice. Thus, neural precursor cells may not only be shaped by microglia, but also regulate microglia functions and activity.

Neural stem cells (NSCs) in the adult mammalian brain can give rise to rapidly dividing NPCs, which produce neurons, astrocytes and oligodendrocytes and functionally contribute to cognition and repair processes after injury1. Transplanted NPCs have beneficial effects on recovery from CNS trauma or disease2,3, but the mechanisms of action remain unclear. Although there is increasing evidence as to how local environmental cues, including microglia and secreted proteins4, influence NPC functionality, few studies have investigated whether NPCs secrete signaling factors, and influence their own environment5. In support of this idea, tissue-specific stem cells outside the brain have been shown to secrete various cytokines, chemokines and other intercellular signaling proteins6, and systemically administered NPCs taking residence in draining lymph nodes can modulate dendritic cell activity in models of multiple sclerosis7.

As we investigated the interactions of NPCs with other cell types in vivo, we observed that microglia were more densely populated and proliferative in neurogenic niches and appeared to be closely associated with NPCs, suggesting that endogenous microglia and NPCs are well positioned to interact with each other (Fig. 1). Thus, to gain a broader view of the communication between cells in the neurogenic niche, we measured 58 secreted signaling proteins on commercially available multiplex antibody-based immunoassays in cell culture supernatants collected from mouse primary microglia, astrocytes, neurons and NPCs. Of the 46 factors that were detectable, each cell type secreted a distinct profile of signaling factors, as visualized by unbiased cluster analysis (Fig. 2a). We identified several factors that were secreted in relatively large amounts by NPCs, including tissue inhibitor of metalloproteinase type-1 (TIMP-1), vascular endothelial growth factor (VEGF) and haptoglobin (Supplementary Fig. 1a). Notably, several of these factors are known to be immunomodulatory proteins and, in particular, prominent regulators of microglia8,9. Thus, we hypothesized that NPCs may not only be regulated by microglia, as suggested by others4, but may, in turn, be capable of regulating microglia. We therefore tested whether NPC-derived factors can modulate major cellular functions of microglia.

Microglia retain the ability to proliferate throughout adulthood, particularly in response to brain injury10. Conditioned medium from primary NPCs induced an increase in the number of BV2...
Figure 2 NPC secret a distinct profile of signaling proteins and regulate microglial functions. (a) Heat map of the secretory profiles of primary NPCs, neurons, astrocytes and microglia based on unsupervised clustering of immunoassay measurements of the listed proteins. Yellow shades represent increased expression of proteins relative to other cell types; blue shades, decreased. (b–d) Conditioned medium from primary mouse NPCs (NPC CM) induced microglial proliferation (b), chemotaxis (c) and phagocytosis (d) (n = 3–6 wells per condition). (e–h) Mice receiving intrastriatal transplants of NPCs (e,f) or NPC conditioned medium (g,h) showed significant increases in the numbers of Iba-1−, Iba-1+ CD68+ and Iba-1+ BrdU+ cells compared with vehicle-treated controls (n = 10 mice per group). Representative merged confocal images of brain sections from mice injected with vehicle and NPCs (e) or unconditioned medium and NPC conditioned medium (g) stained for Iba-1 and CD68 are shown. Scale bars represent 50 μm.

When compared with our cell culture data, injection of NPCs resulted in increases in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with vehicle-treated controls (Fig. 2a,b,d). These data suggest that NPCs, and NPC-secreted factors, can regulate microglia activation in vivo.

To investigate the interaction of NPCs and microglia in vivo, we stereotactically injected either primary mouse NPCs (analyzed for their purity and differentiation potential; Supplementary Fig. 3) or vehicle into the striatum of 2-month-old C57BL/6 mice. Consistent with our cell culture data, injection of NPCs resulted in increases in the number of Iba-1− microglia, with more of these cells being activated and expressing the lysosomal marker CD68 (Iba-1− CD68+), and more microglia proliferating (Iba-1− BrdU+) (Fig. 2e–f and Supplementary Fig. 4a). In addition, we analyzed microglial activation by morphological characterization of Iba-1+ cells. Consistent with our CD68 and BrdU data, we found significantly higher numbers of microglia with activated morphology in striata receiving NPC transplants than in those receiving vehicle controls (P < 0.01; Supplementary Fig. 4b). Notably, conditioned medium from primary mouse NPCs injected into the striatum was sufficient to cause a similar increase in the number of activated microglia (Fig. 2g,h) and Supplementary Fig. 4c). These data suggest that NPCs, and NPC-secreted factors, can regulate microglia activation in vivo.

We next sought to identify a specific NPC-secreted immune factor involved in this regulation. VEGF was highly secreted by NPCs relative to microglia or other CNS cells (Supplementary Fig. 1a), confirming earlier findings1, and has been shown to induce proliferation and chemotaxis in microglia9. We tested whether this factor is sufficient and necessary to mimic part of the effects of NPC-derived factors in our in vitro and in vivo experiments. Indeed, treatment of cultured microglia with recombinant VEGF protein induced proliferation, migration and phagocytosis (Fig. 3a–c). We injected recombinant VEGF protein into the right striatum, and saline into the left striatum as a control, of mice and examined microglia close to the injection sites. Injection of VEGF protein resulted in an increase in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with saline injection (Fig. 3d,e). We injected recombinant VEGF protein into the right striatum, and saline into the left striatum as a control, of mice and examined microglia close to the injection sites. Injection of VEGF protein resulted in an increase in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with saline injection (Fig. 3d,e). We injected recombinant VEGF protein into the right striatum, and saline into the left striatum as a control, of mice and examined microglia close to the injection sites. Injection of VEGF protein resulted in an increase in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with saline injection (Fig. 3d,e). We injected recombinant VEGF protein into the right striatum, and saline into the left striatum as a control, of mice and examined microglia close to the injection sites. Injection of VEGF protein resulted in an increase in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with saline injection (Fig. 3d,e). We injected recombinant VEGF protein into the right striatum, and saline into the left striatum as a control, of mice and examined microglia close to the injection sites. Injection of VEGF protein resulted in an increase in the number of Iba-1+ cells, Iba-1+ CD68+ cells and Iba-1+ BrdU+ cells compared with saline injection (Fig. 3d,e).
knockdown and control conditioned medium (scrambled NPC conditioned medium) into the right and left striatum, respectively. Knocking down VEGF attenuated the effects of NPC-conditioned medium on microglia (Fig. 3h.i and Supplementary Fig. 4g).

To summarize, we found that NPC-derived secreted factors, including VEGF, and transplanted NPCs are powerful regulators of their environment, specifically of microglia. NPCs may also utilize such secreted proteins to self-regulate; for example, stem cell factor, which is also highly expressed by NPCs relative to other cell types, is known to regulate hematopoietic stem cells. Together with previous findings that NPCs express several major components of the B lymphocyte receptor and the observation that transplanted NPCs tend to exert repair functions in injury models and are capable of phagocytosis, it is tempting to speculate that NPCs have a role in maintaining tissue integrity and immune function in the CNS apart from producing neural cells.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

The authors wish to thank S. Villeda and R. Wabl for assistance with the neural progenitor cell cultures and M. Lochrie and the Stanford Neuroscience Gene Vector and Virus Core for generating the shRNA lentiviruses (supported by National Institute of Neurological Disorders and Stroke P30 core grant, NS069375-01A1). This work was supported by grants from the Department of Veterans Affairs (T.W.-C.) and US National Institutes of Health Institute on Aging (R01 AG027505, T.W.-C.), a California Initiative for Regenerative Medicine Award (T.W.-C.), a National Science Foundation predoctoral fellowship (K.I.M.), a Ruth L. Kirschstein NARSAD predoctoral fellowship (1 F31 AG040877-01A1, K.I.M.), the Swiss National Science Foundation (PP6EB-117034 and FASMPF-132321/1, R.H.A.), the Evelyn L. Neizer Fund (R.H.A.), the American Heart Association (AHA 083527N, R.G.), and the Bechtel Foundation (R.G.).

AUTHOR CONTRIBUTIONS

K.I.M. performed in vitro and in vivo experiments and analyzed data from in vivo experiments. R.H.A. performed in vitro experiments and analyzed data. T.W.-C. performed in vitro experiments. G.B. assisted with the VEGF shRNA experiment. M.H.-M. and Y.H. provided conceptual advice and input. K.I.M. and T.W.-C. wrote the manuscript. R.G. and T.W.-C. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nn.3233. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
Online Methods

Cell culture. We isolated and cultured primary mouse NPCs from cortices of postnatal day 0 (P0) C57BL/6 mouse pups as previously described. Briefly, brains were dissected and incubated in DMEM (Gibco) containing 2.5 U ml−1 papain (Worthington Biochemicals), 250 U ml−1 DNase I (Worthington Biochemicals) and 1 U ml−1 dispass II (Boehringer Mannheim) at 37 °C for 45 min. Tissue pieces were washed twice in DMEM with 10% fetal bovine serum (FBS, vol/vol, Gibco) and cellular pellets were resuspended in Neurobasal A supplemented with B27 without vitamin A (Gibco), EGF (20 ng ml−1, PeproTech), FGF-2 (20 ng ml−1, PeproTech) and 1% penicillin-streptomycin (vol/vol, Gibco). To passage cells, neurospheres were harvested by centrifugation, dissociated in Hanks-based cell–dissociation buffer (Gibco), and grown in the medium described above. A rat NPC line was obtained from T. Palmer (Stanford University) and cultured as previously described.

We obtained primary microglia and astrocytes from P0–3 C57BL/6 mouse pups using a previously described protocol, but with some modifications. Briefly, cortices were isolated and stripped of meninges and dissociated with a 25-μg needle. The cell suspension was seeded into poly-L-lysine–coated (Sigma-Aldrich) T75 tissue culture flasks and maintained in DMEM/F12 with 10% FBS and 1% penicillin-streptomycin for 2 weeks to grow a mixed astrocyte/microglia population. We then applied an antigen-antibody–mediated magnetic cell-sorting assay to isolate high-purity microglia and astrocytes (adapted from ref. 17 and Miltenyi Biotech). The mixed glial population was resuspended in MACS buffer (phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (wt/vol) and 2 mM EDTA, pH 7.2) and incubated with CD11b MicroBeads (Miltenyi Biotech). The cell suspension was passed through a magnetic cell separator column (Miltenyi Biotech) fitted into a QuadroMACS cell separator and washed to isolate the unlabeled, purified astrocyte population. The column was then removed from the magnetic separator and flushed with MACS buffer to collect the purified microglia population. The purity of each primary cell type was assessed by immunocytochemistry (data not shown).

The microglia BV2 cell line was obtained from E. Blasi (Università di Modena e Reggio Emilia) and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. We isolated primary hippocampal neurons from 16-d-old CF1 mouse embryos as described previously. Briefly, single-cell suspensions obtained from hippocampi were plated on 24-well culture plates coated with poly-l-lysine at a density of 3 × 10^5 cells per well in DMEM/F-12 with 10% FBS and 1% penicillin-streptomycin and subsequently maintained in Neurobasal medium containing 2% B27 supplement (vol/vol) plus retinoic acid (Gibco).

Cell supernatant collection (conditioned media). We seeded 10^6 mouse primary NPCs at passage 5–7 in 1 ml of culture medium in 12-well plates and collected conditioned medium 48 h later. We cultured 10^6 rat NPCs in 0.5 ml of medium and collected the conditioned medium 24 h later.

For the proteomic analysis of secreted proteins from primary neural cells, we plated 10^5 cells per well in 1 ml of Neurobasal A with B27 on coated 12-well plates and collected supernatants 48 h later. To examine the effects of NPC-conditioned medium on the levels of proteins secreted from microglia, we followed the experimental scheme outlined in Supplementary Figure 2a. The supernatants from microglia treated with or without 10% NPC-conditioned medium (diluted in Neurobasal A) were collected after 48 h.

Proteomic analyses. The relative concentrations of signaling molecules were measured in supernatants described above using antibody-based multiplex immunoassays (Luminex) by Rules Based Medicine, a fee-for-service provider. Measurements were obtained from at least three independent samples.

Proliferation assay. To assess microglia proliferation, we treated 2 × 10^3 BV2 cells per well (six wells per condition) in a 96-well plate with NPC-conditioned medium or recombinant mouse VEGF 164 (R&D Systems) at varying concentrations. Neurobasal A with B27 was used for all dilutions. After 48 h, we stained all cells with Hoechst 33342 (Molecular Probe) and counted the number of cells in each well using an automated imaging system (Cellavista Analyzer, Roche). Data are from multiple independent experiments.

Chemotaxis assay. To test chemotactic activity, we added NPC-conditioned medium or VEGF at varying concentrations to the bottom chamber of 96-well chemotaxis systems (Chemotx chemotaxis system, 101-8sp, Neuroprobe) and either BV2 cells or primary microglia (2 × 10^5 cells per well, five wells per condition) to the top compartment (Neurobasal A with B27 was used for all dilutions). After 3 h, we spun down cells that had migrated through the filter into the bottom chamber, as described by the manufacturer. We removed the filter and stained the cells in the bottom chamber with Hoechst 33342. We then counted the number of migrated cells in each well using the Cellavista imaging system. Data are from multiple independent experiments.

Phagocytosis assay. We analyzed phagocytic function using a previously described assay. We treated BV2 cells or primary microglia (2 × 10^5 cells per well, three wells per condition) in 12-well plates with NPC-conditioned medium or VEGF for 2 h. We then added 2 × 10^5 microspheres (flash-red, 6 μm; Bangs Laboratories) to each well and incubated the cells and microspheres for 1 h at 37 °C on a rotating platform. Prior to being added, the microspheres were opsonized by incubation in 50% FBS/PBS for 30 min at 37 °C. We stopped the assay by adding ice-cold PBS to the cultures, then collected, washed and resuspended the samples in FACS buffer (PBS with 2% FBS and 0.1% sodium azide, vol/vol) in preparation for flow cytometry. We analyzed the samples for fluorescence (PerCP-Cy5.5, phagocytosed microspheres) and light scatter (cell size) to quantify microglia with microspheres. Data are from multiple independent experiments.

Mice. Wild-type C57BL/6J mice were used for stereotactic injections and primary cell cultures (The Jackson Laboratory). Transgenic mice expressing EGF in resident microglia were used to analyze the neurogenic niche; in these mice, the EGF reporter gene was placed into the Cx3cr1 locus encoding the chemokine receptor Cx3cr1 (B6.129P-Cx3cr1^tm1Litt/J, the Jackson Laboratory). Mice were kept on a 12-h light/dark cycle and allowed free access to food and water. All animal care and use complied with the Animal Welfare Act and was in accordance with institutional guidelines and approved by the VA Palo Alto Committee on Animal Research and the institutional administrative panel of laboratory animal care at Stanford University.

Regional microglial expression analyses. To study microglia expression patterns, we administered 5′-bromo-2′-deoxyuridine (BrdU, 100 mg per kg of body weight; dissolved in 0.9% NaCl (wt/vol) to 25 mg ml⁻¹ Sigma-Aldrich) by intraperitoneal injection once a day for 3 d to 2-month-old male wild-type C57BL/6J mice and killed the mice 1 d after injections. The brains were processed and analyzed as described below. Two independent experiments, n = 3–5 mice per group.

NPC transplantation. We dissociated neurospheres into single cells and resuspended the cells at a density of 1 × 10^5 cells ml⁻¹ in culture medium. We performed intrastriatal transplantation in 2-month-old male C57BL/6J mice by infusing 2 × 10^5 viable cells in 2 μl of saline or, in control animals, 2 μl of saline alone into the right hemisphere. Briefly, anesthesia was induced and maintained with 2% isoflurane (vol/vol) in N₂O:air (80:20). Body temperature was monitored and maintained at 37 °C. After fixation of the head in a stereotactic frame (Stoeling Instruments), a midline incision was made to expose the cranium. A frontal burr hole was drilled and a cannula (30G) was stereotactically inserted into the striatum (+0.1 mm anterior, ±2.5 mm lateral, −3.5 mm ventral; relative to bregma). The cell suspension was slowly infused at a rate of 0.5 μl min⁻¹ using a micro-infusion system (UltraMicroPump III, World Precision Instruments). The cannula was left in place for additional 3 min and slowly withdrawn. The wound was sutured and the animal allowed to recover. We administered BrdU 24 h before stereotactic injections and immediately afterwards and killed the mice 24 h after surgery. Two independent experiments, n = 10 mice per group.

NPC-conditioned medium injections. We injected 5 μl of conditioned medium from primary mouse NPCs, or, in control animals, unconditioned medium, into the right striatum, as described above. Two independent experiments, n = 10 mice per group.

VEGF protein and VEGF immunodepleted conditioned medium injections. For injection of VEGF protein, we stereotactically infused 2 μl of recombinant...
VEGF protein (diluted to 200 ng μl\(^{-1}\) in saline, R&D Systems) into the right striatum, as described above. As a control, injections of 2 μl vehicle were performed on the left side (\(n = 5\) mice per group).

For VEGF immunodepletion of NPC conditioned medium, a protein G immunoprecipitation kit (Roche Applied Science) was used according to the manufacturer's instructions. Briefly, we incubated 5 μg of a monoclonal antibody to VEGF (Millipore, 05-443) with 1 ml of NPC conditioned medium for 60 min at 4 °C and then incubated the mixture with a protein G–agarose suspension for 3 h. Agarose beads with the immunoprecipitate were cleared by centrifugation and the supernatant removed. As a control, conditioned medium was processed in an identical way, but without the addition of the antibody. Immunodepleted and undepleted media were intrastriatally infused in the left and right striatum, respectively, as described above. Two independent experiments, \(n = 10\) mice per group.

**VEGF shRNA NPC-conditioned medium injections.** VEGF knockdown in primary NPCs was performed using a lentiviral shRNA expression system (pSHH-H1 shRNA Cloning and Lentivector Expression system, System Biosciences) according to the manufacturer's instructions. Three VEGF-targeted sequences were cloned into the pSHH-H1-copGFP vector (VEGF I, 5′-CCGAGATATTCCGAGTGTTACA TA-3′; VEGF II, 5′-CCGAGATATTCCGAGTGTTACATCATTCAA-3′; VEGF III, 5′-AGTAC TATATATTACCCCTT-3′). The constructs were packaged into viral particles by the Stanford Neuroscience Gene Vector and Virus Core. Conditioned media viruses or a control scrambled sequence (5′-GAATCATCAGGCGTACAGA-3′) were transduced into NPCs infected with the shRNA viruses or a control scrambled sequence (5′-GAATCATCAGGCGTACAGA-3′). The efficacy of the VEGF shRNA knockdown in NPC conditioned medium was analyzed by ELISA (Mouse VEGF DuoSet; R&D Systems), according to the manufacturer's instructions. VEGF shRNA I was used for all subsequent experiments. We stereotactically infused 5 μl of conditioned medium from NPCs infected with VEGF shRNA (VEGF shRNA NPC conditioned medium) or control scrambled shRNA (scrambled NPC conditioned medium) into the right and left striatum, respectively, as described above (\(n = 7\) mice per group).

**Immunohistochemistry.** Immunostaining was performed as described previously\(^{22}\). Briefly, mouse brains were immersed in 4% paraformaldehyde (wt/vol) overnight at 4 °C and sectioned at 40 μm on a freezing microtome. Sections were incubated in 2 N HCL at 37 °C for 30 min for BrdU antigen retrieval, washed in Tris-buffered saline with Tween, blocked with 10% goat serum (vol/vol), and incubated overnight at 4 °C with primary antibodies (rabbit antibody to Iba-1, 1:1,000, WAKO Chemicals, 019–19741; mouse antibody to BrdU, 1:500, Abcam, ab6326; rat antibody to CD68, 1:250, Serotec, MCA1957). For fluorescent stains, primary antibody binding was detected by incubating with corresponding secondary antibodies (Alexa Fluor 488, 546 and 633, 1:500, Molecular Probes, A-11001, A-11003 and A-21094, respectively). Nuclei were counterstained with DAPI (1 mg ml\(^{-1}\), AnaSpec) and the slices were mounted on glass slides with FluorSave (Calbiochem). 3,3′-Diaminobenzidine (DAB, Sigma-Aldrich) stains (Fig. 1a,b) were performed with an ABC labeling kit (Vector Laboratories).

For regional analyses of microglia expression patterns in DAB stains, Metamorph imaging software (Universal Imaging) was used to quantify the percentage of pixels above background staining (thresholded area) in a cortical region of equivalent size in five coronal sections. Velocyt software (PerkinElmer) was used for three-dimensional reconstruction with a coarse algorithm and deconvolution of z stack fluorescence images.

**Data and statistical analyses.** Statistical analyses were performed with GraphPad Instat 3.05 and Prism 5.0 software (GraphPad Software). Means between two groups were compared with two-tailed, paired or unpaired Student's t tests. Comparisons of means from multiple groups against one control group were analyzed with one-way ANOVA and post hoc Dunnett’s multiple comparison test. All experiments were conducted in a randomized and blinded fashion.

To generate proteomic heat maps, we normalized immunoassay measurements of the listed proteins and clustered them using an unsupervised clustering algorithm (Gene Cluster 3.0; M. de Hoon, University of Tokyo)\(^{27}\) to group across sample types and proteins (any proteins that were reported to be undetectable in two out of three measurements for a sample were removed from the analysis before data processing). Java TreeView 1.0.13 (A.J. Saldanha, Stanford University) was used to visualize the heat maps\(^{28}\).

---

15. Reynolds, B.A. & Rietze, R.L. Nat. Methods 2, 333–336 (2005).
16. Giuliani, D. & Baker, T. J. Neurosci. 6, 2163–2178 (1986).
17. Marek, R., Caruso, M., Rostami, A., Grinspan, J.B. & Das Sarma, J. J. Neurosci. Methods 175, 108–118 (2008).
18. Blasi, E., Barluzzi, R., Bocchini, V., Mazzola, R. & Bistoni, F. J. Neuroimmunol. 27, 229–237 (1990).
19. Brionne, T.C., Tesser, I., Masliah, E. & Wyss-Coray, T. Neuron 40, 1133–1145 (2003).
20. Steinkamp, J.A., Wilson, J., Saunders, G. & Stewart, C. Science 215, 64–66 (1982).
21. Giuliani, D., Chen, J., Ingeman, J., George, J. & Noponen, M. J. Neurosci. 9, 4416–4429 (1989).
22. Paxinos, G. & Franklin, K.B.J. The Mouse Brain in Stereotaxic Coordinates (Academic Press, 2001).
23. Buckwalter, M.S. et al. Am. J. Pathol. 169, 154–164 (2006).
24. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. Nat. Methods 9, 671–675 (2012).
25. Soltys, Z., Ziaja, M., Pawlinski, R., Setkowicz, Z. & Janeczko, K. J. Neurosci. Res. 63, 90–97 (2001).
26. Streit, W.J., Walter, S.A. & Pennell, N.A. Prog. Neurobiol. 57, 563–581 (1999).
27. de Hoon, M.J., Iino, S., Nolan, J. & Miyano, S. Bioinformatics 20, 1453–1454 (2004).
28. Saldanha, A.J. Bioinformatics 20, 3246–3248 (2004).