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Modulation of neural stem/progenitor cell proliferation during experimental Herpes Simplex encephalitis is mediated by differential FGF-2 expression in the adult brain

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Abstract

Neural stem cells (NSCs) respond to inflammatory cues induced during brain injury and are thought to be involved in recovery from brain damage. Little is known about NSC response during brain infections. The present study evaluated NSC proliferation during Herpes Simplex Virus-1 brain infection. Total numbers of nestin(+) NSCs increased significantly in infected brains at 6 d post infection (p.i.). However, by 15 d p.i. the nestin(+) population decreased significantly below levels observed in uninfected brains and remained depressed through 30 d p.i.. This initial increase in NSC population occurred concurrently with increased brain cell proliferation, which peaked at 3 d p.i.. On closer examination, we found that while actively proliferating Sox2(+) NSCs increased in number at 6 d p.i., proliferating DCX(+) neuroblasts contributed to the increased response at 3 d p.i.. However, overall proliferation decreased steadily from 15 d p.i. to below control levels. To determine the mechanisms involved in altering NSC proliferation, neurotrophin and growth factor expression profiles were assessed. FGF-2 gene expression increased at 5 d p.i. and was robustly down-regulated at 15 d p.i. (>1000 fold), which was further confirmed by increased FGF-2 immunostaining around the lateral ventricles. Furthermore, supplementing infected animals with recombinant FGF-2, at 15 d p.i., significantly increased the number of proliferating brain cells. These findings demonstrate that the temporal changes in NSC proliferation are mediated through the regulation of FGF-2 and that the NSC niche may benefit from supplementation with FGF-2 during HSV-1 brain infection.

Keywords

Herpes Simplex encephalitis; neural stem cells; proliferation; FGF-2; adult neurogenesis

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Introduction

Herpes Simplex encephalitis (HSE) is the most common cause of sporadic viral encephalitis in the United States. While acyclovir treatment is a highly effective means to control HSV-1 brain infection (Whitley, 2006); greater than fifty percent of human patients with HSE develop life altering neurological deficits (Kimberlin, 2007; McGrath et al., 1997). These neurological deficits are the result of severe brain damage manifesting as reactive astrogliosis and necrosis (Webb SJ, 1989) which in human infections manifests with significant vascular changes with neuronal loss during early stages of disease and the development of astroglial scarring later in infection (Whitley, 2006). The volume of glial scarring correlates to the level of neurological impairment, although the origins of HSV-1-mediated neurological damage and scar formation remain unclear.

In the murine model of experimental HSE, which closely resembles the human disease, immune responses to HSV-1 brain infection begins with infiltration of neutrophils and macrophages between 3 and 5 d p.i.. T cells infiltrate between 6 and 8 d p.i. and peak between 14 and 16 d p.i. with significant persistence beyond 30 d p.i. (Armien et al., 2010; Marques et al., 2008). However, viral antigen is undetectable at 15 d p.i.. The role of persistent T lymphocytes at this stage of disease is unknown; however the presence of activated immune cells is indicative of on-going inflammation that facilitates development of damage and resultant neurological deficits (Armien et al., 2010). Similar studies in the murine model of Japanese encephalitis and experimental autoimmune encephalitis (EAE) have found that CD8(+) T lymphocyte mediated inflammation may be responsible for brain damage and development of long term sequelae (Steinman, 2001; Wang et al., 2003).

A small population of endogenous brain cells called neural stem/progenitor cells (NSCs) play a significant role in adult neurogenesis (Lichtenwalner and Parent, 2005; Parent et al., 2006). NSCs are maintained within distinct germinal niches, which support their multipotent phenotype and self-renewal properties. Adult neurogenesis is composed of highly complex and temporally non-linear progression of cell differentiation that begins with proliferation and ends with integration into existing brain circuitry. Three NSC sub-populations have been shown to proliferate during physiological adult neurogenesis: neural stem cells (B cells), transit-amplifying cells (C cells), and migratory neuroblasts (A cells) (Brown et al., 2003; Doetsch et al., 1997). Previous studies have shown that B/C cells (neural stem/progenitor cells) are immunoreactive for nestin, Sox2, *mushashi*, and *oct4* although levels of expression of these markers may depend on cell progression through differentiation (Kempermann, 2011). Doublecortin (DCX) is the primary cell marker expressed in A cells (Brown et al., 2003; Doetsch et al., 1997; Kempermann, 2011). Brain injury and its resultant inflammation initiate neurogenesis by altering the niche microenvironment to stimulate proliferation and NSC migration (Merkle et al., 2004). NSCs express numerous receptors that respond to immune signals which influence proliferation, direct migration to sites of damage and determine the fate of these pluripotent cells in the injured brain (Chu et al., 2004; Matsuoka et al., 2003).

Inflammation plays a complex role in the post-injury recovery of the CNS. It has been shown that T cells specific to CNS antigens promote recovery from brain injury, provided their activity is controlled (Hauben et al., 2000; Hofstetter et al., 2003; Yoles et al., 2001). However, during EAE, persistent neuroinflammation has been shown to abrogate proliferation of endogenous NSCs and neuroblast migration (Pluchino et al., 2008). In fact, neurogenesis in several brain damage models, including acute ischemic injury (Arvidsson et al., 2002), neurotoxins (Faiz et al., 2005; Tattersfield et al., 2004), and degenerative diseases like Alzheimer's and Parkinson's disease, have varying temporal neurogenic profiles (Capone et al., 2007), potentially directed by relatively unique inflammatory environments.

The mechanisms instructing NSCs to maintain an undifferentiated state or differentiate within an inflamed tissue are poorly understood, particularly following viral encephalitis.

The objective of the present study was to evaluate NSC proliferation in the context of HSV-1 brain infection and its associated inflammation. Given the chronic inflammatory response observed during HSE, we hypothesized that NSC proliferation may be impaired during HSE which ultimately may contribute to the development of neurological deficits.

Materials and Methods

Virus and Infection

HSV-1 brain infection that manifests as acute encephalitis in mice was performed as previously described (Marques et al., 2008). Briefly, HSV-1 strain Syn17+, a neurovirulent strain of HSV, was used in all experiments. The virus was propagated in rabbit skin fibroblasts (CCL68; American Type Culture Collection) and titrated using standard plaque assay. Eight to ten week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were infected with 2.5×10^5 plaque forming units (PFU) by intranasal instillation in anesthetized animals as previously described (Marques et al., 2006).

Transplantation of luciferase(+) NSC

Embryonic NSCs were isolated from the cortices of E14.5 BALB/c mice expressing luciferase (kindly provided by Dr. James Lokensgard, University of Minnesota). The luciferase(+) Balb/c transgenic mice were generated by backcrossing FVB mice expressing luciferase under the control of a CAGGs-CMV promoter into the Balb/c background for ten generations. Luciferase positive (luc(+)) NSCs (90% nestin(+) by flow cytometry) were expanded in culture and transplanted by stereotaxic implantation into the subventricular zone (SVZ), within the striatum, of MHC-matched, non-transgenic BALB/c mice using previously described methods (Ni et al., 2004). Briefly, Luc(+) NSCs (10^6 cells in 3 μ L serum free medium) or 3 μ L serum-free medium control was transplanted into the right SVZ using specific stereotaxic coordinates: rostrocaudal vector [AP]=0mm, interaural vector [ML]=+1.2mm and the dorsoventral vector [DV]= -3.0mm; delivered over a period of 7–10 minutes. The number of luc(+) NSCs to be transplanted was determined by measuring the minimum number of luc(+) cells necessary to provide consistent measurable baseline bioluminescent signal 7 d post transplantation, when recipient mice were infected by intranasal instillation of 2.5×10^5 PFU HSV-1 (Syn17+). By 7 d post-transplant, surgery-induced neuroinflammation had subsided and did not affect establishment of and mortality from HSV-1 brain infection. Bioimaging for luciferase expression in transplanted cells was performed using an *in vivo* imaging system, IVIS50 (Xenogen/Caliper Life Sciences, Alameda, CA) equipped with a charge-coupled camera device, as previously described (Marques, 2008). Briefly, 150 μ g of D-luciferin (Gold Biotechnology) was administered to anesthetized mice by i.p. injection. Animals were imaged 5–10 min after D-luciferin administration and data were acquired using a 5-min exposure window. Signal intensity of luciferase expression, as measured by the total amount of transmitted light, was quantified as a photons/sec/cm² using LivingImage (Caliper Life Sciences, Alameda CA) and Igor (Wavemetrics, Portland, OR) image analysis software. Change in bioluminescence was used as a measure of the numbers of luc(+) NSCs at indicated time points.

Flow Cytometric Quantification of Endogenous Neural Stem Cells

Mouse brain regions from -1 to +3 mm Bregma, which includes the neurogenic regions in the brain, was isolated using a coronal brain matrix (Braintree, Braintree, MA), and a papain based neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA) was used to generate a single cell suspension. Myelin was depleted using myelin depletion beads (Miltenyi, CA).

Live cells were enumerated, and 5×10^5 cells were immunostained for CD45 and nestin (BD Biosciences, San Jose, CA), Ki-67 (Abcam, Cambridge, MA) or SRY-related HMG box-gene (Sox)-2 (eBioscience, San Diego, CA) expression. For absolute quantification of immunostained cells expressing these markers, 50 μ L blank AccuCount particles (Spherotech, Lake Forest, IL) were added to samples immediately before analysis on the flow cytometer (BD FACSCanto). Absolute numbers of each cell population was calculated per the manufacturer's instruction, as a ratio of CD45(-)nestin(+), CD45(-)Ki-67(+), or CD45(-)Ki-67(+)Sox2(+) events to number of AccuCount particles counted.

Immunohistochemistry

Mice were deeply anesthetized using a mixture of ketamine and xylazine and perfused intracardially with 4% paraformaldehyde. Brains were post fixed in 4% paraformaldehyde for 24 hours and equilibrated in 30% sucrose. Fixed equilibrated tissue was frozen in OCT under liquid nitrogen vapor and sectioned at 30 μ m thickness onto gelatin-coated slides. Coronal sections thus obtained were quenched in a 0.3% peroxide solution for ten minutes and blocked with goat serum (5%) in PBS with 0.5% Triton-X for one hour at 25°C. Primary antibodies (Abs) were incubated in the blocking solution overnight at 4°C. Primary antibodies used were rabbit anti-doublecortin Ab (1:1000; Pierce Biotechnologies, Rockford, IL, and PerkinElmer Tyramide Signal Amplification PLUS Fluorescein, Perkin Elmer, Waltham, MA), rabbit or mouse anti-PCNA Ab (1:50; Abcam, Cambridge, MA), mouse anti-Sox2 or rabbit anti-FGF-2 Abs (1:200, Abcam), rat anti-Ki-67 (1:50, eBioscience), and goat anti-HSV-1 (1:100, ViroStat, Portland, MA). This HSV-1 polyclonal antibody is reactive to both immediate early and late structural antigens. Immunostaining using the Tyramide Signal Amplification kit utilized anti-rabbit IgG conjugated to horseradish peroxidase as secondary antibody. Fluorescein was used in the tyramide-horseradish peroxidase reaction step of the kit. Double immunostaining was performed following the Tyramide Signal Amplification kit using the Mouse-on-Mouse Immunohistochemistry kit (Vector Laboratories, Burlingame, CA) for mouse anti-PCNA Ab (1:50; eBioscience, San Diego, CA), or mouse anti-Sox2 Ab (1 μ g/mL; R&D Systems, Minneapolis, MN). The primary antibody incubation was followed with a pan-mouse IgG biotinylated Ab, and an AlexaFluor 555 streptavidin Ab was used as the detecting antibody in this kit. Coronal brain sections were imaged using a Nikon ACT fluorescent microscope/camera system.

PCNA was used as a marker for IHC analysis of NSC proliferation, as an alternative to Ki-67, to identify cells in the S phase of the cell cycle. Sox2 expression was used as a surrogate marker of NSCs to confirm the findings observed within nestin(+) cell populations. Expression of these markers around the lateral ventricles is considered to be specific to NSC (Kempermann, 2011). Immunostaining for NSC phenotypes was quantified exclusively around the SVZ. PCNA or Sox2 immunostaining was not observed in other brain regions including around HSV-1 induced lesions.

Quantification of IHC

To quantify immunostained brain cells, ten sections from each brain were collected 320 μ m apart from (-1 to +3mm bregma) and double immunostained as described in the previous section. Images were collected using identical microscope and camera settings within a specific marker (i.e. PCNA or DCX) at 100X magnification. These images were analyzed using FIJI software (NIH, Bethesda, MD) to create merged images showing cell markers against the DAPI nuclear counterstain. The total numbers of single positive and double positive cells were counted on all ten sections from each brain, located around the sub-ventricular zone of the lateral ventricles and are presented as the average total number of cells per brain of all animals within a group.

RNA isolation and RT-PCR

The neurogenic niche was isolated from infected and sham-infected mice as described above. Single cell suspensions from individual brains were generated using a papain-based neural tissue dissociation kit (Miltenyi Biotec, Auburn, CA). Myelin was depleted as described before and the CD45(−) cell population was isolated by negative selection using CD45 selection beads (Miltenyi Biotec, Auburn, CA). Purity of the CD45(−) cell population was verified by flow cytometry (90% purity), and total RNA was isolated from them using the Qiagen RNeasy kit (Valencia, CA). For the initial screening, 2 µg of total RNA (260/280 ratio between 1.95 and 2.05) from CD45(−) cells harvested from three uninfected and three 15 d p.i. animals. To assess average changes in gene expression from infected and uninfected controls brains, RNA was pooled within the groups and was analyzed by two PCR arrays to assess expression profiles of genes involved in Neurogenesis and Neural Stem Cell Pathway and Neurotrophin/Neurotrophin Receptor genes (SABiosciences; Valencia, CA). Individual gene expression profiles were confirmed by real time RT-PCR was performed on whole brains isolated from intact hemispheres of infected or sham-infected mouse brains at various times post infection (p.i.) by using the Aurum Fibrous and Fatty Tissue RNA isolation kit (Bio-Rad, Hercules, CA) in a SYBR green based PCR reaction. 2 µg of total RNA was used to synthesize cDNA using the RT² First Strand kit (Qiagen, Valencia, CA). Primers used in the single RT-PCR reactions were: Lif forward 5′-acggcaacctcatgaaccaga-3′, reverse 5′-ctagaaggcctggaccaccac-3′; noggin forward 5′-cagatgtggctgtgtgtcac-3′, reverse 5′-gcaggaaacttacactcgg-3′; Transforming Growth Factor-β forward 5′-gactctccactgcaagaccat-3′, reverse 5′-gggactggcgagccttagtt-3′; Fibroblast Growth Factor-2 forward 5′-gcgaccacacgtcaaacta-3′, reverse 5′-tcccttgatagacaaactctc-3′; and Neurotrophin-3 forward 5′-ggagttgccggaagactctc-3′, reverse 5′-gggtgctctgtaatttcctta-3′.

FGF-2 Administration

Infected animals at 15 d p.i. or uninfected controls were given either 1 µg recombinant carrier-free bovine FGF-2 (R&D Systems, Minneapolis, MN) in 1 µL saline or 1 µL vehicle only by intracerebroventricular (i.c.v.) injection into the lateral ventricles (−0.9mm anterior/posterior, +0.5mm midline, and −2.5mm dorsal/ventral relative to bregma). Brains from experimental animals were collected 3 days later, and the neurogenic region was isolated (as in the previous section describing the quantification of endogenous NSCs) for analysis by flow cytometry for CD45 and nestin or CD45 and Ki-67 to assess stem cell numbers and proliferation of brain cells.

Statistics

Student's T test was used to determine differences between groups. P values below 0.05 were considered to be significant. Analysis of Variance (ANOVA) was used for analysis of RT-PCR data.

Results

Transplanted NSCs in the adult brain respond to HSV-1 infection

To evaluate if HSV-1 infection altered neural stem/progenitor cell (NSC) proliferation in the adult brain, embryonic NSCs cultured from E14.5 luc(+) transgenic mice were transplanted into the striatal SVZ of MHC-matched recipient animals and evaluated only as a measure of proliferation. Infected animals exhibited temporally distinct changes in levels of bioluminescence from transplanted luc(+) NSCs at various times post infection (Fig. 1). A robust increase in bioluminescence was observed at 3 d p.i., which was greater than that observed in uninfected control animals (Fig. 1A). This increase was sustained until 15 d p.i.,

when bioluminescent signal decreased precipitously until it reached steady state at 20 d p.i. to levels observed in uninfected animals (Fig. 1A and B). *In vitro* analysis of luc(+) NSCs demonstrated that increased bioluminescence correlated with increased stem cell proliferation and total cell numbers (data not shown). These findings suggest that NSC exhibit an active proliferative response to HSV-1 brain infection during the acute phase of disease and that the increased cell numbers may not be sustained during the chronic phase.

Temporal divergence in endogenous NSC numbers following HSV-1 brain infection

To determine if endogenous NSCs proliferated in response to HSV-1 brain infection, changes in the nestin(+) NSC population were evaluated at 3, 6, 10, 15, and 30 d p.i. by flow cytometry. To exclude myeloid cells from the analysis, brain cells were concurrently immunostained with CD45 and the CD45(−)nestin(+) cell population was analyzed. Compared to uninfected controls, the proportion of nestin(+) NSCs among CD45(−) brain cells increased by 35% at 6 d p.i. from $17.77 \pm 2.00\%$ to $24.00 \pm 5.76\%$ ($p = 0.05$). However, this increase was transient and by 10 d p.i. the percentage decreased to $6.35 \pm 0.63\%$ ($p = 0.01$) and remained at that level through 30 d p.i. (Fig. 2A). To further confirm the observed changes in the nestin(+) population during HSE, absolute numbers of CD45(−)nestin(+) cells were quantified against a fixed number of AccuCount particles added to each sample during flow cytometric analysis (Fig. 2B). The changes observed were preserved in the absolute quantification of total nestin(+) cell numbers. By 15 d p.i., the size of the nestin(+) NSC population decreased by approximately 33% of control, which was sustained through 30 d p.i.. These findings demonstrate that endogenous nestin(+) NSCs exhibit temporally divergent changes in population size, with a robust increase in the nestin(+) cells during the acute phase of HSV-1 brain infection, and a persistent decrease in the size of this stem cell population late in the infection.

Decrease in NSC numbers is not due to virus infection of stem cells during HSE

To ascertain if the decrease in NSCs could be explained by the HSV-1 infection of these cells, coronal brain sections from infected animals at 8 d p.i. were immunostained for both HSV-1 proteins and for representative brain cell phenotype markers. This specific time point was chosen based on previous studies in our laboratory demonstrating peak HSV-1 replication at 8 d p.i. in this murine model (Marques et al., 2008). HSV-1-infected cells in the brain shown by immunostaining for viral antigens were negative for Sox2 staining (Fig. 3A), while Sox2(+) cells in the subventricular zone were negative for viral antigens (Fig. 3B). Viral antigens were predominantly found in MAP-2(+) mature neurons (Fig. 3C) and in Tuj-1 (+) immature neurons (Fig. 3D). Tuj-1 is a neuronal specific class III β -tubulin expressed in immature neurons that precedes the expression of neurofilament protein and MAP-2 during differentiation (Memborg and Hall, 1995; Mutnal et al., 2011; Ni et al., 2004). Tuj-1 and Map-2 (+) neurons were seen in cortical areas of the brain, distal from the SVZ and the NSC niche. Virus-infected GFAP positive astrocytes were not detected in brain sections obtained from the three animals tested (Fig. 3E) but occasional presence of O4⁺ infected oligodendrocytes were observed (Fig. 3F). HSV antigens were undetectable by IHC in infected brains at 30 d p.i. (Armien et al., 2010).

Temporal changes in endogenous brain cell proliferation during HSE

To determine if changes in the nestin(+) NSC population were associated with concomitant alteration in brain cell proliferation, Ki-67 expression in CD45(−) brain cells was evaluated. An increase in the ratio of Ki-67(+) cells was observed at 3 d p.i. ($54.37 \pm 3.54\%$ vs. $43.03 \pm 4.89\%$, infected vs. uninfected respectively) which decreased steadily through 30 d p.i. to $1.57 \pm 0.14\%$ ($p = 0.01$; Fig. 4A). Most notably, the percentage of proliferating cells decreased significantly at 10, 15, and 30 d p.i. ($12.90 \pm 2.07\%$, $p = 0.05$; $8.46 \pm 2.87\%$, $p = 0.05$; $1.57 \pm 0.14\%$, $p = 0.01$ respectively) far below uninfected control levels ($43.03 \pm 4.89\%$). The

absolute numbers of CD45(–)Ki-67(+) per brain, quantified using AccuCount particles, decreased 20-fold from 3 to 30 d p.i. (from $106,031 \pm 1628.1$ Ki-67(+) cells to $5,745 \pm 1094$ cells per brain respectively, $p = 0.01$; Fig. 4B). These findings indicated a robust proliferative response in the brain early after infection at 3 d p.i. (approximately a two-fold increase), but severely reduced proliferation later during the infection. Since the nestin(+) NSC population increased only at 6 d p.i., these findings also suggest that the increase in proliferation occurring at 3 d p.i. may be mediated by a nestin(–) cell population, such as transient amplifying migratory neuroblast cells (A cells), which are also involved in neurogenesis associated with injury (Doetsch et al., 1997).

To assess if NSC proliferation contributed to increased stem cell numbers, brain cells were co-immunostained with Sox2 and Ki-67 at 3, 6, and 15 d p.i., along with uninfected control brains, and analyzed by flow cytometry. These data revealed that trends observed by assessing nestin immunostaining were preserved, independent of analysis method (Fig. 5A–D). Notably, the total number of Sox2(+) cells was decreased at 3 d p.i. (Fig. 5E), but approximately 80% of the total Sox2(+) cells were proliferating (Ki-67(+)) at 6 d p.i. (Fig. 5F). However, both the total number of Sox2(+) and Ki-67(+)Sox2(+) cells decreased significantly at 15 d p.i. This suggests that while the brain initially responds to HSV-1 infection with robust NSC proliferation, by the time viral infection is resolved and no detectable viral antigen is seen in the brain, NSC proliferation is suppressed below physiological levels.

NSCs proliferative responses occur in the sub-ventricular zone of the lateral ventricles

We next proceeded to determine the neuroanatomical location of proliferative responses in HSV-1 infected brains by IHC and to determine the phenotype of NSCs located in the sub-ventricular zone, proliferating in response to viral infection. While the entire brain section was evaluated by IHC, PCNA(+)Sox2 populations were identified only within the SVZ. PCNA(+)Sox2(+) cell populations were quantified at 3, 6, and 15 d p.i. (Fig. 6B–E). Sox2 immunostaining confirmed that peak stem cell proliferation occurs at 6 d p.i., and that the initial increase in proliferation at 3 d p.i., measured by Ki-67 flow cytometry may result from expansion of other progenitor cell populations. Sox2(+) cells were located predominantly in the SVZ. Interestingly the total number of Sox2(+) cells decreased at 3 d p.i. (124.0 ± 10.1 total cells per infected brain vs 194.5 ± 27.3 total cells per control brain, $p = 0.05$; Fig. 6F), even though the number of PCNA(+) Sox2(+) stem/progenitor cells remained unchanged at this time point (37.0 ± 2.0 total cells per infected brain vs 21.5 ± 0.7 total cells per control brain). However, both the total number of Sox2(+) and proliferating Sox2(+) cells were significantly increased at 6 d p.i. (269.5 ± 19.11 total cells per infected brain, $p = 0.01$, and 69 ± 4.02 total cells per control brain, $p = 0.01$). As expected both total Sox2(+) and proliferating Sox2(+) cell numbers, at 15 d p.i., decreased significantly compared to uninfected brains levels (total Sox2(+), 175.00 ± 9.02 total cells per infected brain, $p = 0.05$; double positive 20 ± 4.04 total cells per infected brain, $p = 0.01$). These findings show that NSC proliferation increased at early points during HSV-1 brain infection, but decreased significantly at later points.

The initial proliferative response is observed in doublecortin(+) migratory neuroblasts

Coronal brain sections were immunostained for doublecortin (DCX) and PCNA to determine if neuroblasts around the SVZ proliferated in response to HSV-1 infection (Fig. 7A–F). Proliferating DCX(+)PCNA(+) cells were significantly increased at 3 d p.i. (69.5 ± 14.1 total cells per infected brain vs 23 ± 2.1 total cells per control brain, $p = 0.01$). Although the number of proliferating DCX(+) neuroblasts around the SVZ contracted at 6 d p.i. their numbers were larger than seen in uninfected animals (Fig. 7G). However, during the chronic phase of infection (15 d p.i.), the number of proliferating DCX(+) cells

decreased significantly relative to uninfected controls (15.5 ± 2.97 total cells per infected brain vs 23 ± 2.1 total cells per control brain, $p = 0.01$). These findings suggest that both Sox2(+) stem cells and neuroblasts respond to HSV-1 infection in a temporally distinct manner (Table 1), and viral infection results in a significant decrease in proliferation of both neural stem and progenitor cells at 15 d p.i..

Differential expression of genes associated with neurogenesis at 3 and 15 d p.i

To further investigate the mechanisms that decrease NSC proliferation, a preliminary screen of 153 genes associated with neurogenesis was performed on mRNA isolated from CD45(−) brain cells from infected or uninfected animals at 15 d p.i.. Gene expression was analyzed on two PCR arrays (Neurogenesis/Neural Stem Cell Pathway and Neurotrophin/Neurotrophin Receptors arrays, SABiosciences; data not shown) from which, five genes selected based on change in expression (≥ 2 fold) compared to uninfected controls. Expression of leukemia inhibitory factor (LIF), noggin, transforming growth factor- β (TGF- β), neurotrophin-3 (NT-3), and fibroblast growth factor-2 (FGF-2) was further analyzed to determine if these selected genes exhibited differential expression at 3 and 15 d p.i. (Fig. 8). LIF and noggin were down regulated while TGF- β was up regulated at both time points examined. However, NT-3 and FGF-2 were differentially regulated at 3 and 15 d p.i. NT-3 was down regulated by 2.00 ± 0.77 fold at 3 d p.i. and up-regulated by 4.76 ± 0.84 fold at 15 d p.i.. Conversely, FGF-2 expression increased by 2.52 ± 0.73 fold at 3 d p.i., but decreased 1342.84 ± 33.33 fold at 15 d p.i.. Since FGF-2 has been shown to induce stem cell proliferation (Dayer et al., 2007), the role of FGF-2 in altering NSC proliferation during HSE was further investigated.

Differential kinetics of FGF-2 gene and protein expression during acute and chronic HSE

To evaluate if FGF-2 played a role in altering NSC proliferation during HSE, the kinetics of FGF-2 gene expression at 3, 5, 11, and 15 d p.i. was first analyzed in whole brain samples (Fig. 9A). Consistent with the data demonstrating increased brain cell proliferation at 3 and 6 d p.i., FGF-2 gene expression was up-regulated during the acute phase of infection (2.52 ± 0.73 and 3.44 ± 0.34 fold respectively), and down-regulated during the chronic phase of infection at 11 and 15 d p.i. (288.35 ± 13.33 and 1342.84 ± 33.33 fold respectively).

To verify that reductions in mRNA expression were reflected by altered FGF-2 protein expression, brain sections from uninfected and infected animals at 3, 6 and 15 d p.i. were immunostained for FGF-2 and visualized by fluorescent microscopy (Fig. 9B). Consistent with the RT-PCR analysis, there was more FGF-2(+) immunofluorescence at 3 and 6 d p.i. around the subventricular zone, compared to the 15 d p.i. animals (coronal sections examined from -1 to $+2$ mm bregma). Interestingly, there was FGF-2 immunostaining around the meninges, which also decreased at 15 d p.i. compared to uninfected controls (data not shown). These findings demonstrate that FGF-2 expression in infected brains correlates with observed changes in endogenous brain cell proliferation during HSE. These data also suggests that the decreased levels of FGF-2 may be responsible for decreased NSC proliferation at 15 d p.i..

FGF-2 treatment of chronically infected mice increases endogenous brain cell proliferation

To investigate if decreased FGF-2 levels in HSE animals influenced NSC proliferation, HSV-1 infected mice at 15 d p.i. were pulsed with $1 \mu\text{g}$ FGF-2 or saline vehicle by intracerebroventricular (i.c.v.) injection. Brains were collected three days post-treatment and single cell suspensions from the neurogenic region were assessed for changes in CD45(−)Ki-67(+) proliferating brain cells (Fig. 10A) and nestin(+) neural stem/progenitor cells (Fig. 10B) by flow cytometry. In infected FGF-2 treated animals, CD45(−)Ki-67(+) proliferating endogenous brain cell population increased significantly relative to the saline-

treated infected animals and was not significantly different from uninfected controls (Fig. 10A). Although brain cell proliferation increased with FGF-2 treatment of infected animals, no increase was observed in the nestin(+) stem/progenitor cell population in these animals (Fig. 10B). We further characterized this increase in endogenous brain cell proliferation and observed that majority of proliferating cells with FGF-2 treatment in the SVZ were DCX(+) (Fig. 10C). These findings suggest that administration of exogenous FGF-2 in infected animals could stimulate brain cell proliferation and potentially be used to generate new stem/progenitor cells in virus-infected brains.

Discussion

The present study demonstrates that adult murine neural stem/progenitor cells (NSCs) exhibit temporally distinct proliferative responses following Herpes Simplex Virus (HSV)-1 brain infection. In response to infection, luciferase(+) NSCs transplanted into the SVZ displayed robust expansion, indicated by an increase in total bioluminescence, which was sustained until 15 d p.i. This increase in bioluminescence was observed in infected animals, even as luminescence signals decreased in uninfected animals over time, indicative of NSC expansion or increased cell survival resulting from viral brain infection. Bioluminescence signals in infected animals subsequently dropped, to levels observed in uninfected animals, during the chronic phase of infection (>15 d p.i.). Bioluminescence imaging is a powerful non-invasive method to track the behavior of stem cells longitudinally *in vivo*, which when optimized can detect the presence of 3000 to 6000 transplanted cells in the brain (Aswendt et al., 2013). While this method provides insight into acute responses of neural stem cells to HSV-1 brain infection, the fate of these implanted cells is not known.

To expand our understanding of the impact of HSV-1 brain infection on adult neurogenesis, endogenous NSCs, identified by either nestin or Sox2 expression, were analyzed in infected mouse brains. NSC numbers increased at 6 d p.i., while total brain cells proliferating in response to infection increased as early as 3 d p.i. These early proliferating cells at 3 d p.i. were identified as DCX(+) neuroblasts. This initial increase in NSC and neuroblast proliferation was abrogated during the chronic phase of infection and resulted in significantly lower numbers of nestin and Sox2(+) NSCs in the infected brain. Expression of several growth factors was evaluated to determine the mechanisms involved in mediating these temporally divergent NSC responses. Most notably, the change in FGF-2 gene expression in HSV-1 infected brains mirrored the changes in NSC proliferation, suggesting that decreased FGF-2 consequent to infection may lead to suppressed NSC proliferation. Interestingly, a single dose of exogenous FGF-2 in chronically infected animals was sufficient to increase endogenous brain cell proliferation within 3 days post treatment, albeit without net increases in nestin(+) NSCs.

NSC proliferation in response to HSV-1 increased initially after infection. This initial increase in neurogenesis was followed by chronic suppression of proliferation in endogenous NSCs. This “window of neurogenesis” after the initiation of injury is not unique to HSE, but has been reported following non-infectious brain injury, like stroke and traumatic brain injury (Arvidsson et al., 2002; Kernie and Parent, 2010; Zhou et al., 2011). Interestingly, viral brain infections are reported to suppress proliferative response (Das et al., 2010), potentially due to virus infection and lysis of NSCs (Das and Basu, 2008) or is mediated by inflammation resulting from viral infection (Das et al., 2011). Our findings indicate that inhibition of NSC proliferation did not result directly from HSV-1 infection of NSCs, demonstrated by a distinct absence of viral antigen in the SVZ and in Sox2(+) NSCs. We and others have shown that experimental HSV-1 brain infection in mice typically occurs as a highly localized, focal neural tract infection without marked dissemination outside infected neurons in immunocompetent brains (Armien et al., 2010; Himmelein et al., 2011;

Labetoulle et al., 2003). Furthermore, suppression of NSC proliferation, in the present study, was observed at the time when viral replication was not detected in the brain (Armien et al., 2010; Marques et al., 2008). This implies that the effect of HSV-1 on NSC proliferation is spatially and temporally separated, potentially mediated by indirect mechanisms resulting from the host response to infection.

Inflammatory mediators have been shown to influence NSC proliferation, migration and fate differentiation (Gonzalez-Perez, 2010). The characteristics of inflammation in stroke and the early stages of HSV-1 brain infection, where NSC proliferation increases, are driven largely by macrophages and neutrophils (Armien et al., 2010; Arvidsson et al., 2002). These innate immune cells mediate both beneficial and harmful inhibitory effects on tissue regeneration (Dietel et al., 2012; Hu et al., 2012; Savage et al., 2012). On the other hand, acute inflammation following JEV infection, characterized by CD8 T cell infiltration, in addition to macrophages and neutrophils, results in inhibition of NSC proliferation (Das et al., 2011). Similarly, chronic T lymphocyte-mediated inflammation in EAE leads to decreased NSC proliferation and reduction of the NSC population (Ziv et al., 2006) over extended periods following disease induction (Imitola et al., 2004; Khoury et al., 1992). We demonstrate in the present study that the initial increase in proliferative response is followed by a stepwise decrease in both total NSC numbers and proliferation (Table 1) at a time when activated CD8(+) T lymphocytes dominate the inflammatory milieu (Armien et al., 2010; Marques et al., 2008). Activated CD8 T cells are essential for the maintenance of latency in HSV-1 infected neurons, effected through interactions between virus-infected cells and lymphocyte-derived lytic granule components viz. granzyme B (Jiang et al., 2011; Knickelbein et al., 2008). Release of granzyme B from activated CD8 lymphocytes has been shown to impair NSC proliferation and differentiation via the voltage-dependent potassium channel, Kv1.3 (Wang et al., 2010). This suggests that persistent inflammation mediated by CD8(+) T lymphocytes following HSV-1 brain infection may similarly inhibit NSC proliferation thus impairing neurogenesis and brain recovery.

Closer examination of proliferating brain cell phenotypes revealed that multiple cell types compose the initial proliferative response in the brain. This endogenous brain cell response at 3 d p.i. was enabled by an increase in DCX(+) neuroblasts, while Sox2(+) proliferation peaked a few days later at 6 d p.i.. Proliferative response to brain injury has been shown to involve both cell types, which have different functions in the neurogenic process. This initial proliferative response has been shown to occur as the result of macrophage activation independent of the etiology of injury (Doetsch et al., 1997; Hu et al., 2012; Kigerl et al., 2009). A number of studies have demonstrated unequivocally that proliferation of Sox2(+) cells occur early after injury, with few reporting the proliferation of DCX(+) neuroblasts. DCX(+) neuroblasts as the first identifiable proliferating cell type during neurogenesis in the adult brain (Brown et al., 2003), indicative of rapid expansion of these cells post injury. Involvement of DCX(+) neuroblasts have also been shown to be critical for the short-term outcomes associated with recovery from stroke (Jin et al., 2010). Similarly, progressive decrease in the proliferation and numbers of neuroblasts in EAE potentially influences clinical outcomes (Huehnchen et al., 2011; Pluchino et al., 2008; Tepav evi et al., 2011), indicative of the critical role neuroblasts play in recovery from brain injury. In the present study, the depressed proliferative response during the chronic phase of infection, where proliferation of both neural stem and progenitor cells was reduced, suggests clinical intervention at this point may be beneficial similar to that observed in ischemic injury following enhancement of NSC proliferation (Zhao et al., 2007). Additional analysis of restorative neurogenesis in virus-infected brains is essential to demonstrate the contribution the different cell types make in the recovery from HSE-mediated damage.

Chronic inflammation alters the molecular constituents of the tissue microenvironment leading to suppression of NSC proliferation. Studies in EAE (Clemente et al., 2011), stroke (Arvidsson et al., 2003), and neuropathy (DeLeo et al., 1997) have demonstrated that neurotrophic factors, such as FGF-2, glial-derived neurotrophic factor (GDNF), and anosmin-1, are down-regulated during the chronic inflammation that develops subsequent to injury. This contrasts with findings during the acute injury where neurotrophic factors such as brain-derived neurotrophic factor (BDNF), stromal cell-derived factor (SDF)-1 α , and FGF-2 are up-regulated (Imitola et al., 2004; Kiprianova et al., 2004; Shruster et al., 2012). Our study found that during the course of HSV-1 infection and associated injury, NT-3 and FGF-2 gene expression is differentially regulated, increasing during the acute phase and decreasing in the chronic phase of infection.

Decreased expression of FGF-2 in HSV-1 infected mice was associated with concomitant inhibition of NSC proliferation. Although virus infection is spatially separated from the NSC niche in the SVZ, it is known that immune mediated effects following viral infections induce global changes to the tissue microenvironment (Marques et al., 2008; Mutnal et al., 2011). In uninjured brains, FGF-2 production is seen in GFAP(+) cells in the SVZ, which are unique from nestin(+) NSC (Frinchi et al., 2008), but the effects of virus induced inflammation on production of FGF-2 in the SVZ is poorly studied (Russo et al., 2011). Changes in production of immune and brain produced growth factors, particularly FGF-2, have been documented consequent to inflammation resulting from ischemic injury (Cheng et al., 2011; Dayer et al., 2007), multiple sclerosis (Clemente et al., 2011), and Alzheimer's Disease (Kiyota et al., 2011). The types of growth factors and cytokines present in the injured tissue alter the outcomes of neurogenic processes initiated by injury (Haughey et al., 2002; Kalluri and Dempsey, 2008). IL-1 β treatment of brain cell cultures induced FGF-2 production (Bourcier et al., 1995; DeLeo et al., 1997). Production of IL-1 β is seen early during HSV-1 infection (Aravalli et al., 2005) giving credence to the possibility that modulation of FGF-2 expression early during HSV-1 brain infection may be mediated through IL-1 β . Given the association between immune cytokines and growth factor production during neuroinflammation (Bellavance and Rivest, 2012; Kalluri and Dempsey, 2008; Zhu et al., 2012), it is plausible that the dynamic nature of the inflammatory cytokine milieu generated during HSE (Aravalli et al., 2005; Armien et al., 2010; Marques et al., 2006) modulates the functional response of NSCs ultimately defining outcomes associated with injury-induced neurogenesis. We demonstrate that treatment of infected animals with FGF-2 stimulates proliferation of brain cells. Similar results have been described after ischemic brain injury and in Alzheimer's disease models, where exogenous FGF-2 administration stimulates proliferation of NSCs and improves behavioral outcomes. (Feng et al., 2012; Kiyota et al., 2011). The present study demonstrates that a single FGF-2 pulse in the lateral ventricles during the chronic phase of infection is sufficient to promote proliferation of endogenous brain cells, indicating revitalization of neurogenesis suppressed by chronic inflammation.

Conclusions

Our findings demonstrate that HSV-1 infected brains exhibits temporally distinct proliferative responses including evidence of increased neurogenesis early in disease that is progressively arrested during the chronic phase, and that FGF-2 expression rises and falls concomitant with this proliferative response. These divergent responses coincide directly with the constituents of the inflammatory milieu, dominated by macrophages and neutrophils acutely and activated T cells in the chronic phase (Armien et al., 2010; Marques et al., 2008). This implies that any intervention to enhance neurogenesis would need to consider the effect of the inflammatory milieu on NSCs. We demonstrate that supplementing FGF-2 in the chronic phase of HSE enhanced the proliferative response in the brain, a possible first step in the recovery process. Further studies are essential to determine if FGF-2

may be used to offset the neurological deficits seen in the wake of viral infections of the brain.

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Highlights

- Adult murine NSCs exhibit temporally distinct proliferative responses during HSE (80)
- Endogenous NSCs expand acutely but proliferation decreases significantly at 15 d p.i. (85)
- This divergent response coincides with infiltration of different immune cell types (82)
- FGF-2 expression in the infected brain mirrors the trends seen in NSC proliferation (84)
- Addition of FGF-2 in the brain increases proliferation without increasing NSC numbers (85)

Figure 1. NSCs proliferate during HSE

Bioluminescence images obtained from animals receiving MHC-matched, luciferase-expressing (luc^+) NSCs (1×10^6 cells/animal), transplanted into the SVZ by stereotactic injection (coordinates at bregma: 1.2 mm lateral, 0.5 mm caudal and 3.0 mm ventral from the skull surface). Animals were infected with HSV-1 17 syn⁺, 5 d post-transplantation. **(A)** Levels of luciferase expression, indicative of the number of NSCs, were assessed by bioluminescence imaging longitudinally over the indicated time period. Bioluminescence images are presented on a pseudocolor scale showing photon flux values (photons/cm²/sec) at each time point in infected and uninfected animals. Representative images of the same animal (infected) at the indicated time points are shown. **(B)** Average percent change in total cranial luminescence normalized to the luminescence of each animal at time zero (0 d p.i.) is presented in HSV-1-infected (gray bars) and compared to uninfected animals (white bars). Mean values at each time point obtained from 4–6 animals followed longitudinally are presented as dashed lines in the plots and medians are solid lines. Luciferase expression levels were detected up to 40 d post-transplant, the latest time-point tested.

Figure 2. Total number of nestin(+) NSCs increase in the HSV-1 infected brain

Flow cytometry on single cell suspensions from infected or sham-infected Balb/c mouse brains trimmed to include neurogenic regions (−1.98 mm to 3.80 mm relative to bregma) were analyzed for nestin expression in the non-myeloid cell population (CD45(−)) and is shown at the indicated time points during HSV-1 infection. **(A)** Representative dot plots showing nestin and CD45 staining in brain cells demonstrate changes in a ratio of nestin(+) cells in the non-myeloid compartment (top left quadrant) over the course of HSV-1 infection. Quadrant gates were established based on isotype stained brain samples. Ratios of CD45(−)nestin(+) cells, presented as %±SEM, were obtained from 9–12 brains examined per time point. **(B)** The absolute number of nestin(+) cells was calculated from individual brain samples using a fixed number of AccuCount beads in each sample. Total number of live CD45(−)nestin(+) cells per brain was obtained from 3 replicates using 9 animals per replicate at each time point. The pooled average numbers in each group is presented as ±SEM. * p 0.05.

Figure 3. NSCs are spared from HSV infection

Coronal brain sections from HSV-1 infected animals were immunostained for viral antigen and brain cell specific markers at the peak of viral infection in the brain (8 d p.i.) to determine the brain cell types susceptible to viral infection. Photomicrographs demonstrating the presence of viral antigens (green) in the different cell types are presented. (A) Cells expressing viral antigens in the cortex did not express Sox2 (red) while (B) Sox2(+) cells observed in the subventricular zone of the same section were negative for viral antigens. (C) On the other hand, viral antigens (red) were predominantly found in MAP-2(+) neurons (green) (D) and in immature neurons, immunostained with Tuj-1 (green). Nuclei were counterstained with DAPI (blue) and arrow denoting viral positive neurons. (E) Viral infected GFAP(+) astrocytes were not detected in brain sections tested (F) but occasional O4(+) infected oligodendrocytes (arrow) were observed.

Figure 4. Endogenous brain cells initiate proliferation at 3 p.i

Single cell brain suspensions, immunostained for Ki-67 and CD45, were analyzed by flow cytometry. **(A)** Representative dot plots showing Ki-67 and CD45 staining in the brain cells of uninfected or infected animals at 3, 6, 10, 15, and 30 d p.i. demonstrated changes in a percentage of proliferating cells in the non-myeloid compartment (top left quadrant) over the course of HSV-1 infection. Quadrant gates were established based on isotype stained brain samples. Ratios of CD45(-)Ki-67(+) cells, presented in each quadrant as average values (%) $\pm$ SEM, were obtained from 9–12 brains at each time point. **(B)** Absolute numbers of non-myeloid brain cells proliferating in response to HSV-1 infection was determined using counting beads in each brain sample analyzed for Ki-67 expression by flow cytometry. Data presented are representative plots obtained from 3 independent experiments with 9–12 animals per time point. Average values ($\pm$ SEM) from pooled data at each time point are presented. * $p < 0.05$.

Figure 5. Enhanced Sox2(+) NSC proliferation at 6 d p.i. is abrogated at 15 d p.i

Single cell suspensions from mouse brains encompassing regions between -1.0 and $+3.0$ mm Bregma of (A) uninfected and (B–D) infected animals at (B) 3 d p.i. (C) 6 d p.i. or (D) 15 d p.i. was analyzed for Sox2 and Ki-67 expression by flow cytometry. Data presented was based on analysis of CD45(–) brain cell population. Absolute numbers of (E) Sox2(+) and (F) proliferating Ki-67(+)Sox2(+) cells were quantified using counting beads in each brain sample analyzed, as described previously. Data are presented as averages $\pm$ SEM of nine animals per time point. * p 0.05 and ** p 0.01.

Figure 6. Sox2(+) NSC proliferation is increased in the SVZ at 6 d p.i

Infected and sham-treated brains were perfused with fixative and cryosectioned for immunohistochemistry. A total of 10 coronal sections were collected 320 μ m apart from the region between -1.0 mm and $+3.0$ mm relative to bregma. Photomicrographs of brain sections immunostained for Sox2 (red), an NSC marker, and PCNA (green), proliferating cell nuclear antigen are presented. (A) The lateral ventricle (LV) from an uninfected mouse is shown to indicate the neuroanatomical location from which subsequent images (B–E) were obtained. Representative merged images showing Sox2 and PCNA immunostaining in the lateral ventricles from (B) uninfected animals, and infected animals at (C) 3 d p.i., (D) 6 d p.i., (E) 15 d p.i. are presented. Arrow heads show double positive cells. (F) Single- and double-positive cells were counted from all 10 coronal sections obtained from infected and sham-infected brains at the various time points indicated. The total numbers of Sox2 single-positive (black bars) and Sox2/PCNA double-positive (gray bars) cells are presented as the average ($\pm$ SEM) number of cells observed in 10 sections obtained from 3 animals at each time point. * p 0.05 and ** p 0.01.

Figure 7. Doublecortin(+) neuroblasts proliferate at 3 d.p.i

Brain sections processed and prepared as described, in the previous figure were immunostained for PCNA and doublecortin (DCX), a marker for proliferating cells and neuroblasts, respectively. (A) IHC analysis of control brains showing PCNA (left panel; red) or DCX (middle panel; green) staining on a representative coronal section and the merged image (right panel) showing the nuclear localization of PCNA antigen in the cell (arrows indicating double positive cells). (B) The lateral ventricle from a 3 d p.i. infected brain is shown to indicate the neuroanatomical location from which subsequent images (C–F) were obtained. Representative merged images showing patterns of PCNA and DCX immunostaining around the ventricles from (C) uninfected, (D) 3 d p.i., (E) 6 d p.i., (F) 15 d p.i. brains are presented. Arrows showing the presences of double positive cells in the infected brain at 3 and 6 d p.i.. (G) Proliferating DCX(+)PCNA(+) neuroblasts were quantified from coronal sections obtained from infected and sham-infected brains at the various time points indicated (as described above). Increase in the total numbers of DCX(+)PCNA(+) cells in the brain was observed at 3 d p.i. but is significantly decreased at 15 d p.i. relative to uninfected controls. Data presented are averages ($\pm$ SEM) cells counted from 3 animals at each time point. * $p < 0.05$ and ** $p < 0.01$.

Figure 8. NT-3 and FGF-2 are differentially regulated during acute and chronic HSE

To identify mediators involved during the changes observed in NSC proliferation at 3 and 15 d p.i., genes known to affect neurogenesis were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). Five selected genes (Leukemia inhibitory factor (Lif), Transforming Growth Factor- β (TGF- β), noggin, neurotrophic factor-3 (NT-3), and fibroblast growth factor-2 (FGF-2)) were assessed individually from total RNA obtained from whole brains of 3 infected animals at 3 d p.i. (black bars) and 15 d p.i. (gray bars). Data are presented as average fold change ($\pm$ SD), in normalized mRNA expression, from uninfected controls.

Figure 9. Kinetics of FGF-2 expression during HSE

FGF-2 expression was evaluated over the course of HSE. (A) Total RNA isolated from uninfected and HSV-1-infected brains was assayed for FGF-2 mRNA expression at the time points indicated. Change in FGF-2 expression over time was calculated as an average fold change ($\pm$ SEM) from normalized gene expression in uninfected controls ($n=3-5$ per time point). (B–D) To confirm FGF-2 mRNA changes were reflected in protein expression, coronal brain sections from uninfected and infected animals were evaluated by IHC. Representative photomicrographs of coronal brain sections showing the expression of FGF-2 protein around the lateral ventricles in (B) uninfected brains, compared to that observed in infected brains at (C) 3 d p.i., (D) 15 d p.i.. Images are representative of 3 brains assessed within each group.

Figure 10. FGF-2 treatment rescues inhibition of cell proliferation at 15 d p.i

To determine if FGF-2 replacement impacts brain cell proliferation, infected mice at 15 d p.i. were given 1 μ g FGF-2 or saline by i.c.v. injection. At 3 d post treatment, brains were analyzed by flow cytometry for total number of (A) CD45(-)Ki-67(+) proliferating cells and (B) CD45(-) nestin(+). (A) Total numbers of proliferating Ki-67(+) endogenous brain cells reverted to uninfected (control) levels post FGF-2 treatment (FGF-2) compared to HSV-1-infected saline-treated (saline) animals. Data are presented as % of control compared to uninfected (control) animals. (B) Similar analysis of nestin(+) NSC showed no difference in the total numbers whether infected animals were given vehicle (saline) or FGF-2. Data demonstrating differences among treatment groups are presented as average values ($\pm$ SEM) obtained from 5 animals per group. * $p < 0.05$. (C) FGF-2 treatment stimulates proliferation of DCX(+) cells in HSV-1 infected brains. Photomicrographs of coronal brains sections obtained at 15 d p.i from infected, untreated animals (left panel) or infected, FGF-2-treated animals (right panel) and immunostained for DCX (green) and Ki67 (red) are presented. Representative images taken from four animals per treatment group are shown.

Differential NSC proliferation during HSE

Table 1

The table presents observed trends of proliferation by cell marker and total endogenous brain cell proliferation as fold-change compared to the uninfected samples. The fold change values are presented as follows: (-----) 0.3; (----) 0.31 to 0.6; (---) 0.61 to 0.75; (--) 0.76 to 1; (+) 1.01 to 1.25; (++) 1.26 to 1.5; (+++) 1.51 to 2, (++++), 2.01

Cell Markers	3 d p.i.	6 d p.i.	10 d p.i.	15 d p.i.	30 d p.i.
Total Positive Cells					
Sox2(+)	(--)	(++)	nd	(-)	nd
nestin(+)	(--)	(+++)	(--)	(--)	(--)
Doublecortin(+)	(+)	(-)	nd	(++)	nd
Ki-67(+)	(+++)	(++)	(-)	(--)	(-----)
Proliferating Cells					
Sox2(+)	(--)	(+++)	nd	(-----)	nd
Doublecortin(+)	(++++)	(++)	nd	(--)	nd

Fold change values: (-----) 0.3, (----)=0.31 to 0.6, (---)=0.61 to 0.75, (--)=0.76 to 1, (+)=1.01 to 1.25, (++)=1.26 to 1.5, (+++)=1.51 to 2, (++++), 2.01; nd, not determined