Coxsackievirus Preferentially Replicates and Induces Cytopathic Effects in Undifferentiated Neural Progenitor Cells

Running Title: Coxsackievirus Infection of Neural Progenitor Cells

Authors
Ginger Tsueng¹, Jenna M. Tabor-Godwin¹, Aparajita Gopal¹, Chelsea M. Ruller¹, Steven Deline¹, Naili An¹, Ricardo F. Frausto², Richard Milner³, Stephen J. Crocker²,4, J. Lindsay Whitton², and Ralph Feuer¹

Author Affiliations:
¹Cell & Molecular Biology Joint Doctoral Program, Department of Biology, San Diego State University, San Diego, CA, 92182-4614, USA
²Dept. of Immunology and Microbial Science, SP30-2110, The Scripps Research Institute, La Jolla, CA 92037, USA
³Dept. of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla CA 92037, USA
⁴Present Address: Department of Neuroscience, Faculty of Medicine, University of Connecticut Health Center, Farmington, CT 06030-3401

*Correspondence to: Dr. Ralph Feuer
Cell & Molecular Biology Joint Doctoral Program
Department of Biology, San Diego State University
5500 Campanile Drive; San Diego, CA 92182-4614
Phone: 619-594-7377 Fax: 619-594-0777
Email: rfeuer@sciences.sdsu.edu

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ABSTRACT

Enteroviruses, including coxsackieviruses, exhibit significant tropism for the central nervous system, and these viruses are commonly associated with viral meningitis and encephalitis. Previously, we described the ability of coxsackievirus B3 (CVB3) to infect proliferating neuronal progenitor cells located in the neonatal subventricular zone and persist in the adult murine CNS. Here, we demonstrate that cultured murine neurospheres, which comprise neural stem cells and their progeny at different stages of development, were highly susceptible to CVB3 infection. Neurospheres, or neural progenitor and stem cells (NPSCs), isolated from neonatal C57 BL/6 mice supported high levels of infectious virus production and high viral protein expression levels following infection with a recombinant CVB3 expressing eGFP protein. Similarly, NPSCs isolated from neonatal actin-promoter GFP transgenic mice (actin-GFP NPSCs) were highly susceptible to infection with a recombinant CVB3 expressing dsRED protein. Both nestin+ and NG2+ progenitor cells within neurospheres were shown to preferentially express high levels of viral protein as soon as 24 hours post-infection (PI). By day 3 PI, viral protein expression and viral titers increased dramatically in NPSCs with resultant cytopathic effects (cpe) and eventual cell death. In contrast, reduced viral replication, lower levels of cpe, and diminished viral protein expression levels were observed in NPSCs differentiated for 5 or 16 days in the presence of fetal bovine serum (FBS). Despite the presence of cpe and high levels of cell death following early CVB3 infection, surviving neurospheres were readily observed and continued to express detectable levels of viral protein as long as 37 days after initial infection. Also, CVB3 infection of actin-GFP NPSCs increased the percentage of cells expressing neuronal class III β-tubulin following their differentiation in the presence of FBS. These results suggest that neural stem cells may be preferentially targeted by CVB3, and neurogenic regions of the CNS may support persistent viral replication in the surviving host. In addition, normal progenitor cell differentiation may be altered in the host following infection.
INTRODUCTION

Non-polio enterovirus infections are thought to be directly responsible for a majority of clinical cases of viral meningitis and encephalitis in the US every year. An estimated 10-15 million symptomatic enterovirus infections every year may account for up to 75,000 cases of meningitis hospitalizations in the US alone (35). In particular, coxsackievirus B and enterovirus 71 have been routinely identified in patients suffering from viral meningitis. Other serious CNS diseases may result following enterovirus infection, including acute disseminating myelitis (12) and acute transverse myelitis (20). Despite the significance of these viruses in human disease, much remains to be determined regarding their neurotropism, immune activation following infection, and potential long-lasting effects on the central nervous system (CNS) in the surviving host.

We previously described a neonatal mouse model of coxsackievirus B3 (CVB3) infection whereby nestin+ neural stem cells and myeloid cells were identified as the primary target cell during early infection (15) (16) (37). Eventually, many cells infected with CVB3 underwent apoptosis (15). However, host survival was commonly observed in parallel with detectable levels of viral RNA in the adult CNS for at least 90 days post-infection (PI). The ability of CVB3 to persist in other organs, in particular the heart, has been well documented (25) (6) and may involve genetic alterations in the virus which may limit replication and cytopathic effects (cpe) in the host cell (36) (23) (24). We hypothesized that the continued presence of viral RNA and/or viral gene products may affect normal neural stem cell migration and/or differentiation in the developing CNS.

Relatively little is known about the susceptibility of neural stem cells to neurotropic viral infections. Surprisingly, neural stem cells residing in the CNS remain active into adulthood replenishing neurons within the olfactory bulb and dentate gyrus (3) (19). As these neural stem cells give rise to mature neurons, their proliferative and activation status may render them attractive targets for neurotropic viruses. Also, the migratory nature of immature neuroblasts...
may assist in virus dissemination within the brain following infection of progenitor or stem cells (15). Recently, both human cytomegalovirus (27) (7), HIV-1 (31) (34), and Japanese encephalitis virus (9) (10) have been shown to target neural stem cells and may influence stem cell function (26) (30) (29) and immunogenicity (11).

We wished to investigate the ability of CVB3 to infect neural stem cells grown in culture in order to more clearly evaluate the consequences of CVB3 infection on stem cell survival and dysfunction in a less complex environment. Neural progenitor and stem cells (NPSCs) isolated from the brains of one-day old mice form neurosphere aggregates which can be passaged indefinitely in culture. NPSCs, or their differentiated counterparts, were infected with recombinant coxsackieviruses expressing either eGFP (eGFP-CVB3) or dsRED (dsRED-CVB3) and inspected for virus production and alterations in stem cell function. Also, the level of virus replication and virus protein expression in NPSCs was compared to their differentiated counterparts. Our results suggest that virus production and protein expression levels were robust in undifferentiated neurospheres, yet differentiated cells appeared to be refractory to infection and virus protein expression.

Surprisingly, some NPSCs survived infection and supported a “carrier-state” infection provided that cultures were regularly replenished with fresh complete NPSC media. We hypothesize that CVB3 may persist in a similar fashion in vivo within a quiescent subset of neural stem cells. Evaluating CVB3 infection of NPSCs in culture may help to understand factors influencing preferential viral replication in dividing progenitor cells, and these investigations may ultimately illuminate possible chronic alterations in neural progenitor cell differentiation during persistent infection within the surviving host.
**MATERIALS & METHODS**

**Isolation and production of a recombinant coxsackievirus.** The generation of a recombinant coxsackievirus expressing eGFP has been described previously. Briefly, the CVB3 infectious clone (pH3) (obtained from Dr. Kirk Knowlton at University of California at San Diego) was engineered to contain a unique SfiI site which facilitates the insertion of any foreign sequence into the CVB3 genome. The generation of recombinant coxsackievirus expressing the enhanced green fluorescent protein (eGFP-CVB3) and dsRED (dsRED-CVB3) has been described previously (Feuer et al., 2002) (37). Virus titrations were carried out as described previously (14). Viral stocks were prepared on HeLa RW cells maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum. Viral stocks were diluted in DMEM before inoculation.

**Isolation, culture, and infection of neurospheres.** Mouse experimentation conformed to the requirements of the San Diego State University Animal Research Committee and the National Institutes of Health. C57BL/6 mice and actin promoter-GFP transgenic mice were obtained from the Scripps Research Institute animal facilities or Harlan Sprague Dawley (Harlan Laboratories, San Diego CA). Breeding pairs were checked every day. As described previously, NPSCs were derived from isolated cortices of newborn mice, mechanically and enzymatically dissociated, and then plated as a single cell suspension in complete NPSC Media - DMEM/F12 media supplemented with 2% B27 (Invitrogen), 20ng/mL EGF (Invitrogen), 20ng/mL bFGF (Preprotech), 5ug/mL Heparin (Sigma), and 0.5% pen/strep (8). Free-floating neurospheres were separated and transferred into new flasks every two days. Neurospheres were vigorously dissociated and resuspended in NPSC culture medium to a concentration of 10^5 cells/mL in a T-25 flask (BD falcon). Neurospheres were plated onto chamber slides and infected with eGFP-CVB3 at various multiplicities of infection (moi) in NPSC Media. Alternatively, NPSCs were differentiated for 5 or 16 days in Differentiation (Diff.) Media - DMEM supplemented with 1%
FBS, N1 Supplement (Sigma), and 0.5% pen/strep. Following infection, differentiated cells were continuously cultured in either Diff. Media or in NPSC Media. Supernatants were harvested over time to determine viral titers. The percentage of dead cells was determined by trypan blue staining followed by cell counting using a hemacytometer. Neurosphere cultures were replenished with complete NPSC media and passed every three days up to 37 days post-infection (PI). Infected neurospheres were examined over time using fluorescence microscopy.

**Immunofluorescence microscopy.** Live NPSC or differentiated NPSC cultures infected with eGFP-CVB3 or dsRED-CVB3 were imaged using a Zeiss Axio Observer D.1 inverted fluorescent microscope with ApoTome Imaging System. Alternatively, infected NPSCs were fixed in 4% para-formaldehyde and washed three times in PBS. Viral protein expression was determined by native eGFP (green) or dsRED (red) expression. Fixed cells were blocked with 10% normal goat serum (NGS) and immunostained using the following antibodies: Nestin (Covance Inc.; Cat# PRB-315C) at 1:1000, neuronal class III β-tubulin (Covance Inc.; Cat# PRB-435P) at 1:1000, GFAP (Sigma Inc; Cat# G 9269) at 1:1000, NG2 (Chemicon Inc.; Cat# AB5320) at 1:500, Olig2 (Abcam Inc.; Cat# ab33427) at 1:1000, and MBP at 1:1000 (Chemicon Inc.; Cat# AB980). Primary antibodies were diluted in 2% NGS in PBS (150-200 μl per slide) in humidified chamber and incubated overnight. Slides were washed with PBS for 5 min (3X). Secondary antibodies (at 1:1000) conjugated to Alexa-Fluor-594, Alexa-Fluor-488, or Alexa-Fluor-350 were diluted with 2% NGS in PBS (150-200 μl per slide) and incubated overnight. Following incubation with the secondary antibody, slides were washed with PBS for 5 min (3X). Three to five representative images of the cultures were taken for each sampling time point at multiple magnifications.
**Image J Analysis.** For each fluorescent image, three fluorescent channels for each image were exported separately without color overlay. Threshold adjustments were applied to generate a black and white image using NIH ImageJ (public domain software). These black and white images were analyzed using ImageJ to quantify fluorescent signal. In each case, marker expression was normalized prior to applying the Student t-test to determine statistical significance. In C57 BL/6 NPSCs infected with eGFP-CVB3, marker expression was normalized to DAPI. In actin-GFP NPSCs infected with dsRed-CVB3, marker expression was normalized to GFP.
RESULTS

CVB3 infection of primary neural progenitor and stem cells (NPSCs) in culture. We isolated and cultured primary NPSCs from both C57 BL/6 and actin promoter-GFP transgenic mice in order to determine their ability to support CVB3 infection. NPSCs grown in culture formed neurospheres which comprise both stem and progenitor cells. As expected, isolated neurospheres expressed high levels nestin, a marker for neural stem and progenitor cells, as determined by immunofluorescence (data not shown). Neurospheres were infected at a multiplicity of infection (moi) = 0.1 with a recombinant coxsackievirus B3 expressing eGFP (eGFP-CVB3), and viral protein expression (eGFP) was evaluated by immunofluorescence microscopy merged with Hoffman Modulation Contrast (HMC) over the course of seven days (Figure 1).

As early as 1 day post-infection (PI), virus protein expression was observed in a minority of cells within the neurosphere (Figure 1A). An increase in virus protein expression was readily seen in neurospheres by day 2 PI (Figure 1B). By day 4 PI, nearly all cells within many neurospheres expressed high amounts of viral protein (Figure 1C). In contrast, differentiated cells located near the periphery of the neurosphere which had attached to the surface of the chamber slide (most likely following the depletion of growth factors in the complete NPSC media) failed to express appreciable levels of viral protein. Higher magnification of day 4 infected cultures illustrated the lack of viral protein expression in differentiated cells adjacent to heavily-infected neurospheres (Figure 1D). By day 6 PI, the level of viral protein expression dropped dramatically (Figure 1E), and cytopathic effects (cpe) were readily apparent near residual neurospheres (Figure 1F; notched white arrows). After 7 days PI, many infected neurospheres disappeared presumably due to cpe ((Figure 1G; white arrow), and viral protein expression was limited to rounded cells or cellular debris while differentiated cells continued to lack viral protein expression. In contrast, unharmed neurospheres remained visible in mock-infected control cultures grown in parallel for 7 days (Figure 1H; white arrow).
We evaluated the amount of infectious virus produced over time in these infected neurosphere cultures by plaque assay (Figure 1I; dark grey line). Within 2 days PI, viral titers increased dramatically in neurosphere cultures. By 3 days PI, peak production of infectious virus was observed which corresponded closely with viral protein expression levels, as determined by immunofluorescence microscopy. By day 6 PI and beyond, viral titers dropped corresponding to the presence of cpe within the neurosphere cultures. Taken together, these results suggest that virus protein expression and viral replication was robust in stem and progenitor cells found within neurospheres; in contrast, differentiated cells adjacent to infected neurospheres appeared to be refractory to infection.

Also, NPSCs were infected with eGFP-CVB3 at a higher moi (moi = 100.0), replenished with complete NPSC media every third day, and followed for infectious virus production by plaque assay for 16 days PI (Figure 1I; light grey line). High viral titers were observed in these NPSC cultures for up to 16 days pi. Surprisingly, some NPSCs survived initial infection in the presence of replenished complete NPSC media and these infected NPSCs supported a carrier-state infection characterized by a steady-state infection in which many or all of the cells become infected (18). These findings suggest the presence of a subpopulation of stem cells which may be resistant to virus-mediated cpe; a resistant primary stem cell may restore the neurosphere culture and generate additional target cells for the maintenance of virus production over time.

**Preferential infection of nestin + and NG2 + cells in neurosphere cultures.** Although NPSCs consist of nestin + stem cells, cells lacking nestin expression were also found within neurospheres. These nestin - cells may represent more committed progenitor cells or immature neuronal or glial cells. To determine which population of cells within a neurosphere were most susceptible to infection, NPSCs were infected with eGFP-CVB3 (moi = 0.1), harvested two days later, and immunostained for nestin, as well as three additional downstream lineage markers including neuronal (neuronal class III β-tubulin) and glial cell markers (GFAP, NG2). Neuronal
class III β-tubulin is highly expressed within neuroblasts and immature neurons. GFAP is a marker commonly utilized to discriminate immature glial cells and astrocytes (28). NG2 has been used to identify oligodendrocytes precursor cells (4). A Zeiss Axio Observer with Apotome Imaging System was utilized to detect the co-localization of infected cells and cells expressing downstream lineage molecules. The Apotome Imaging System utilizes structured illumination technology allowing for the collection of optical sections and 3D reconstruction.

Both nestin+ and nestin− NPSCs expressed high levels of viral protein (Figure 2A, white arrow and notched blue arrow, respectively). Also, we detected the expression of three downstream lineage markers within infected neurospheres at two days PI (Figure 2A, β-tubulin, GFAP, and NG2 immunostaining). Viral protein expression was observed in cells expressing all three downstream markers, although infection was preferentially within NG2+ cells. Each field outlined by a hatched box showed higher magnification of infected cells expressing each marker (white arrows) or failing to express the marker (notched blue arrows). The number of infected cells expressing each marker was quantified over two days (Figure 2B). Four cell populations were analyzed, and the proportion of infected cells was found to be highest in nestin+ cells at day 1 and day 2 PI (66.7% and 75%, respectively). Also, an increasing percentage of NG2+ cells expressed detectable levels of viral protein at day 1 and day 2 PI (40% and 70.5%, respectively). The least percentage of infected cells was observed in β-tubulin+ cells (9.1% and 14.9; day 1 and day 2 PI, respectively) and GFAP+ cells (10.4% and 14.8%; day 1 and day 2 PI, respectively), although percentage of double-positive cells increased for both populations over the course of two days. These results suggest that CVB3 may be preferentially infecting progenitor cells which follow the oligodendrocyte pathway, or alternatively the differentiation pathway of infected stem cells may be preferentially shifted toward the oligodendrocyte lineage.

Virus replication and cell death in actin-promoter GFP neurospheres infected with dsRED-CVB3. We tested the ability of an additional recombinant coxsackievirus expressing dsRED
(dsRED-CVB3) to infect an independent isolation of neurospheres from actin promoter-GFP transgenic mice (Figure 3). Also, the infection of actin promoter-GFP neurospheres (actin-GFP NPSCs) was carried out at two multiplicities of infection (0.1 and 10.0) and followed over seven days in culture. The eventual fate of infected neural stem cells surviving acute infection and followed via the adoptive transfer into the CNS will be of great interest in future studies involving actin-GFP NPSCs. Actin-GFP NPSCs were highly susceptible to infection with dsRED-CVB3 and supported high levels of viral protein expression (dsRED) at moi = 0.1 (Figure 3A-E), and moi = 10.0 (Figure 3F-J). At moi = 0.1, viral infection of actin-GFP NPSCs led to cpe at day 3 PI (Figure 3C and Figure 3L, white arrows). We observed accelerated cpe at the higher moi in cultures infected with dsRED-CVB3 at day 2 PI (Figure 3G and Figure 3K, white arrows). The high level of virus protein expression (red) in actin-GFP NPSCs was represented by single channel images (Figure 3G, inset; and Figure 3M). In contrast, no cpe was observed in mock-infected actin-GFP NPSCs at any time point (Figure 3N). By 7 days PI, only cellular debris remained for the majority of actin-GFP NPSCs infected at either a high or low moi (Figure 3E and Figure 3J). High viral titers were observed over seven days in actin-GFP NPSCs infected at either a high or low moi (Figure 3O). As expected, viral titers rose quicker in actin-GFP NPSCs infected at a higher moi (Figure 3O, dark grey line with circles).

We inspected the degree of cell death in NPSCs following CVB3 infection over the course of 3 days PI (Figure 4). NPSCs isolated from C57 BL/6 mice were infected with eGFP-CVB3 at two multiplicities of infection (moi = 0.1 or 10.0), and viral protein expression was followed by fluorescence microscopy (Figure 4B and Figure 4C, respectively). Alternatively, actin-GFP NPSCs were infected with dsRED-CVB3 at two multiplicities of infection (moi = 0.1 and 10.0) and compared directly to neurospheres infected with eGFP-CVB3 (Figure 4E and Figure 4F, respectively). Both recombinant coxsackieviruses induced rapid cpe in NPSCs at the greater moi (moi = 10.0) by day 3 PI (Figure 4C and Figure 4F, white arrows). Conversely, the greater level of cpe observed at the higher moi may be due to more cells infected initially, yet
more readily detected by our trypan blue staining protocol. By trypan blue staining, the percentage of dead cells was determined over time for C57 BL/6 NPSCs infected with eGFP-CVB3 (Figure 4G) and for actin-GFP NPSCs infected with dsRED-CVB3 (Figure 4H). An increase in the percentage of dead cells was observed over three days with both recombinant viruses. Also, NPSCs infected with a greater moi (moi = 10.0) showed accelerated and higher levels of cell death by trypan blue staining at day 3 PI, a time point when cpe was observed in cultures by fluorescence microscopy. These results demonstrate that neurospheres from two independent isolation procedures were similarly susceptible to infection and cpe using two recombinant coxsackieviruses.

Reduced virus replication in NPSCs differentiated with FBS. We inspected in greater detail the susceptibility of differentiated cells derived from NPSCs to CVB3. NPSCs were cultured in the presence of FBS in order to increase surface attachment and induce differentiation (Figure 5). Prior to the addition of FBS, NPSCs expressed high levels of nestin; however in the presence of FBS, neurosphere aggregates began to attach to the surface, reduced their expression of nestin, and expressed high levels of neuronal class III β-tubulin (Figure 5A). NPSCs were grown in the presence of FBS for 5 or 16 days, and subsequently cultured with complete NPSC media lacking FBS (Figure 5B). NPSCs cultured in the presence of FBS for 16 days appeared to be in a more differentiated state and attached more robustly to the culture surface, as compared to NPSCs cultured with FBS for only 5 days. Surprisingly, NPSCs differentiated for 5 days in the presence of FBS, and replenished with NPSC media lacking FBS, showed a progressive return to spherical neurosphere morphology at day 3 and day 7 post-treatment. Similarly, NPSCs differentiated for 16 days in the presence of FBS, and replenished with NPSC media lacking FBS also showed substantial morphological changes after 7 days in NPSC media suggesting “de-differentiation” to neurosphere aggregates.
We inspected the expression levels of nestin, GFAP, neuronal class III β-tubulin (β-tubulin), and MBP in NPSCs differentiated for 5 or 16 days in the presence of FBS and replenished with complete NPSC media lacking FBS (NPSC Media), or cultured in the presence of FBS (Diff. Media) (Figure 5C). As expected, NPSCs treated for 5 or 16 days with FBS (5 Day Diff. or 16 Day Diff., respectively) and cultured in Diff. Media expressed high levels of GFAP, β-tubulin, and MBP. Although nestin continued to be expressed in 5 Day Diff. cultures in Diff. Media, the cellular distribution of signal was altered (diffuse rather than filamentous). In contrast, 16 Day Diff. cultures in Diff. Media failed to express detectable levels of nestin, suggesting that these cells were well differentiated (Figure 5C; white arrows). Paralleling the morphological results, 16 Day Diff. cultured in NPSC Media were induced to express moderate to high levels of nestin suggesting “de-differentiation”, similar to what has been described previously (2) (21).

5 Day Diff. or 16 Day Diff. NPSC cells were infected with eGFP-CVB3 and cultured either in NPSC Media or Diff. Media. Virus protein expression levels were evaluated by fluorescence microscopy up to 10 days following infection (Figure 6). As determined previously, undifferentiated neurospheres cultured in NPSC media and infected with eGFP-CVB3 supported robust levels of virus protein expression and cpe, although surviving neurospheres were observed at day 7 and day 10 PI (Figure 6; NPSCs + NPSC Media, white arrows). The least amount of virus protein expression was observed in 16 Day Diff. NPSCs continuously cultured with FBS (Figure 6; far right column), although detectable levels of virus protein expression were eventually observed at day 10 PI within attached cells with stretched morphology. Also 5 Day Diff. NPSCs cultured continuously with FBS (Figure 6; column second from right) expressed relatively low levels of virus protein. Little to no cpe was observed in both of these differentiated cultures continuously cultured with FBS.

In 16 Day Diff. NPSC cultures replenished with NPSC Media, virus protein expression was observed at day 3, and continued to day 10 PI in the presence of low levels of cpe. In
contrast to differentiated NPSCs continuously cultured with FBS, 5 Day Diff. NPSC cultures replenished with NPSC Media supported detectable virus protein expression levels very early (day 1 PI). At day 3 PI, these cultures expressed high levels of virus protein in the presence of cpe (day 3 PI). The attached cells with a differentiated, stretched morphology in 5 Day Diff. cultures replenished with NPSC Media eventually gave rise to neurosphere aggregates with a spherical morphology, most likely reflecting “dedifferentiation” in these cells following the removal of FBS. The majority of 5 Day Diff. cultures replenished with NPSC Media underwent cpe by day 7 PI; however, surviving neurospheres were observed in these cultures (Figure 6; 5 Day Diff. + NPSC Media, white arrow).

The morphology of many infected cells in 5 Day Diff. cultures replenished with NPSC Media suggested infection of immature neurons with long axonal extensions expressing high levels of virus protein (Figure 7A, white arrows). Higher magnification showed the axonal processes of an infected cell extending and connecting to uninfected neighboring cells in these cultures (Figure 7B and Figure 7C, white arrows). In some cases, two infected neuronal cells with axonal processes made contact with uninfected cells separating both infected cells, suggesting preferential infection in cells with axonal morphology Figure 7D. Also, the presence of cellular “blebbing” was occasionally seen in adherent cells infected with CVB3, suggesting ongoing apoptosis in these cultures (Figure 7E and Figure 7F, notched blue arrows). Despite the high degree of cpe in the 5 Day Diff. cultures replenished with NPSC Media, surviving neurospheres could be observed at day 10 and day 37 PI, suggesting “dedifferentiation” of the cultures and the presence of stem cells resistant to virus-mediated cpe, and capable of renewing the neurosphere cultures (Figure 7G and Figure 7H, notched blue arrows). A closer inspection of neurospheres from day 37 PI indicated the continued expression of detectable levels of virus protein at these extended time points ((Figure 7I and Figure 7J, notched blue arrows), indicative of viral persistence in this long-term culture.
We measured viral titers in NPSCs differentiated with FBS, as compared to undifferentiated NPSCs up to 7 days PI. Viral titers were evaluated in 5 Day Diff. cultures either continuously treated with FBS, or replenished with NPSC media (Figure 7K). The highest viral titers (over $10^7$ pfu/ml at day 2 PI) were observed in NPSCs cultured in NPSCs media over all time points. 5 Day Diff. cultures replenished with NPSC media supported lower levels of virus replication at all time points, as compared to infected NPSCs. The least amount of virus replication was observed in 5 Day Diff. cultures continuously treated with FBS. These results match the virus protein expression levels observed in Figure 6, and suggest preferential CVB3 replication in NPSCs, as compared to their differentiated counterparts. A greater difference in viral replication was observed in 16 Day Diff. cultures continuously treated with FBS or replenished with NPSC media (Figure 7L). Viral titers were undetectable in 16 Day Diff. cultures continuously treated with FBS until day 7 PI. Also, 16 Day Diff. cultures replenished with NPSC media supported very low levels of infectious virus, as compared to infected NPSCs. These results suggest that the longer NPSCs are differentiated in the presence of FBS (5 days versus 16 days), the less these cells support CVB3 replication.

We analyzed viral protein expression levels and viral titers in NPSCs during the course of well-defined differentiation to determine the susceptibility of the three neural cell lineages to CVB3 infection (Figure 8). 5 Day and 16 Day Diff. cultures were infected with eGFP-CVB3 at a low (moi=0.1) and high moi (moi=1.0). Diff. Media was added back to infected cultures, and cells were harvested after a period of time (day 3 and 14 PI, respectively) based on the kinetics of viral protein expression, as determined in Figure 6. We evaluated infection by eGFP expression and the degree of NPSC differentiation utilizing nestin and three differentiation markers (GFAP, β-tubulin, and MBP) (Figure 8A). For comparison, 5 Day Diff. cells were infected with eGFP-CVB3 at a low moi (moi=0.1), cultured in the presence of NPSC Media, and stained for nestin or differentiation markers at day 2 PI. As shown in Figure 6, 5 Day Diff. + NPSC Media cultures showed relatively high levels of viral protein expression at day 2 PI. Furthermore, 5 Day Diff.
NPSC Media cultures expressed high levels of nestin as well as the three differentiation markers, suggesting that these cells were not fully differentiated. 5 Day Diff. + Diff Media cultures showed less viral protein expression and more diffuse nestin staining. Higher magnification images of hatched white boxes for 5 Day Diff. + Diff Media cultures demonstrated colocalization of viral protein expression and either nestin⁺ or β-tubulin⁺ cells (Figure 8A; white arrows). Also, we observed a lack of viral protein expression in 5 Day Diff. + Diff Media cultures expressing high levels of GFAP or MBP (Figure 8A; notched blue arrows). For the single time point examined before harvest (Day 3 PI), low to moderate levels of viral titers were observed in 5 Day Diff. + Diff Media cultures (Figure 8B), depending upon the initial viral inoculum. In contrast, 16 Day Diff. + Diff Media cultures showed no viral protein expression at 14 days PI, suggesting again that well-differentiated cells did not support CVB3 replication (as shown in Figure 6). Furthermore, these cultures lacked nestin expression and expressed moderate to high levels of GFAP, β-tubulin, and MBP, which suggested their highly differentiated status. Also, low viral titers were observed until day 11 PI for cultures given a higher initial inoculum (moi=1.0) (Figure 8C). These results suggest that the highly differentiated 16 Day Diff. cultures supported significantly less CVB3 replication, as compared to 5 Day Diff. cultures.

Infection of NPSCs and alteration of the differentiation pathway. We inspected the ability of infected neurospheres to differentiate into the three downstream cell lineages following treatment with FBS. C57 BL/6 NPSCs were mock-infected or infected with eGFP-CVB3 (moi = 0.1). In parallel, actin-GFP NPSCs were mock-infected or infected with dsRED-CVB3 (moi = 0.1). After two days PI, the NPSC cultures were treated with FBS (Differentiation Media) for an additional three days (Figure 9). Infected NPSC cultures were observed for virus protein expression by fluorescence microscopy before (day 2 PI) and after (day 3 PI) FBS treatment. Intriguingly, infected NPSCs appeared less attached or flattened in the presence of FBS at day 3 PI, as compared to mock-infected NPSCs. By fluorescence microscopy, infected NPSCs were
shown to express detectable levels of viral protein at day 2 and day 3 PI. After three days of FBS treatment (day 5 PI), infected NPSC cultures were fixed and stained for three neural cell lineage markers (GFAP, β-tubulin, and Olig2). Olig2 expression has been utilized previously in other studies to identify cells in the oligodendrocyte lineage (5). The amount of fluorescent signal for each marker was quantified by ImageJ analysis, and the relative fluorescence was calculated as a percentage of the total number of cells for each stain. For C57 BL/6 NPSCs, DAPI was utilized to calculate relative fluorescence values. For actin-GFP NPSCs, GFP signal was utilized to calculate relative fluorescence values.

We compared the relative fluorescence value for each marker between mock and infected NPSCs differentiated with FBS. For C57 BL/6 NPSCs, no statistical significant changes were observed in the relative fluorescence levels for all three markers following infection. In contrast, a statistically significant increase in relative β-tubulin levels ($p=0.02$; Students T-test) was observed in actin-GFP NPSCs following infection, as compared to mock-infected control cultures. Also, the percentage of GFAP$^+$ cells was reduced in actin-GFP NPSCs following infection, although not by a statistically significant level ($p=0.06$; Students T-test). No statistically significant change was observed in the relative fluorescence levels of Olig2 within actin-GFP NPSCs following infection. A direct comparison of actin-GFP and C57 BL/6 NPSCs was problematic due to the methodology applied in obtaining the ratios of the representative markers. For example, the downstream cell lineages for actin-GFP NPSCs were determined based on cytoplasmic GFP expression, as compared to nuclear stain (DAPI) for C57 BL/6 NPSCs. Alterations in the relative population of downstream cell lineages following differentiation of infected NPSCs may be dependent upon isolation differences during stem cell harvesting, isolation and cell culture, as well as potential stochastic differences inherent during infection and after the FBS treatment. Also, infected NPSCs were reduced in number at 5 days PI as compared to mock-infected NPSCS, suggesting either cpe or a reduction in cellular...
proliferation during the differentiation procedure. Each downstream progenitor cell lineage may be differentially susceptible to CVB3-induced cpe. These results indicate that CVB3 may bias neural stem cell differentiation by increasing the percentage of immature β-tubulin⁺ neuroblasts.
DISCUSSION

The ability of CVB3 to target neural stem cells in the neonatal CNS raises many questions regarding stem cell function and normal brain development within the surviving host. We previously established a murine model for neonatal coxsackievirus B3 (CVB3) infection, and proliferating nestin+ progenitor cells were identified as primary targets for early infection (15) (16). In addition, CVB3 established a persistent infection in mice surviving a neonatal infection, characterized by the continued detection of viral RNA by RT-PCR, along with long-lasting lesions and chronic signs of inflammation and microgliosis up to 9 months following infection (17).

To more easily discriminate the effects of CVB3 infection on neural stem cell function and differentiation, we thought it imperative to duplicate any findings observed *in vivo*, in parallel with neural stem cells grown in culture. Given that CVB3 is a cytolytic virus, we wished to determine if neural stem cells were susceptible to infection and exhibited cytopathic effects (cpe) following infection. Therefore, we inspected the susceptibility of neurospheres, or NPSCs grown in culture, to CVB3 infection. Also, we wished to determine whether CVB3 infection might alter the differentiation path of NPSCs. The benefits of using NPSCs grown in culture include the ability of using a relatively pure population of target cells, controlling the multiplicities of infection (moi) at the onset of infection, and discriminating the initial time of infection during time course assays - factors difficult to control *in vivo*.

Despite the benefits of examining infected NPSCs grown in culture, we realized that neurosphere aggregates form relatively complex mixtures of both stem and progenitor cells expressing a variety of cell markers. In fact, infection of cultured primary NPSCs may be a particularly intriguing and dynamic model of CVB3 infection given the complex mixture of cell types with potentially differential antiviral responses induced during infection. In this regard, little is known about the ability of stem cells to induce and respond to interferon following infection. We expect that the interferon response within our primary stem cell culture system may be
responsible for the observed carrier-state infection observed in NPSCs, which may ultimately parallel the establishment of viral persistence within the CNS. Also, our results suggest that susceptibility to infection may reflect the heterogeneity of progenitor cells in neurosphere aggregates; each progenitor cell type may have a slightly different susceptibility to coxsackieviral infection and cpe depending upon the progenitor type or stage of differentiation. Also, some adherent cells with a flattened, stretched morphology remained in culture and appeared to be resistant to infection, as judged by viral protein expression. Adding to the complexity, susceptibility to infection may be altered as progenitor cells differentiate into the downstream lineages. A recent publication suggests preferential coxsackievirus replication within immature neurons expressing relatively high levels of CAR, as compared to their fully differentiated counterparts (1).

Two independent neurosphere isolations from C57 BL/6 mice and actin-promoter GFP transgenic mice generated NPSCs which were shown to be highly susceptible to CVB3 infection. Also, virus-mediated cell death in NPSCs following CVB3 infection was observed by trypan blue staining. The ability of CVB3 to infect neonatal NPSCs and induce cpe may shed light on potential CNS development defects following neonatal infection. In addition, possible long-term consequences may result in the surviving host, given the substantial number of functional neural stem cells in the adult CNS. However, the capacity of adult NPSCs to support CVB3 infection remains to be determined. Also, distinct populations of NPSCs from different regions of the murine CNS may be differentially susceptible to infection. Our previous published in vivo data suggests that NPSCs in the SVZ and hippocampus are both susceptible to infection (15) (16). However, the degree of susceptibility and the ability to establish a persistent infection may be reflected by the particular anatomical location of isolated NPSCs.

Despite clear cpe in infected NPSCs following CVB3 infection, surviving neurospheres were observed in cultures replenished with complete NPSC media. These results suggest that NPSCs may mount functional antiviral responses, perhaps by inducing, and/or responding to
interferon following infection (38). In this respect, very little is known regarding the ability of stem cells to induce innate immune responses in response to pathogens. It remains unknown if prolonged antiviral responses or chronic interferon may alter or compromise normal neural stem cell function and the proper generation of downstream cell lineages. The possibility remains that a particular nestin+ stem cell population may exist within the neurosphere aggregate which is both resistant to CVB3 infection yet can re-establish the NPSC culture. Nestin+ progenitor cells have been previously shown to be responsible for neurosphere regeneration in culture (32).

Our results also indicate the ability of CVB3 to persist in NPSCs grown in culture. This finding may be particularly relevant given our recent work indicating the ability CVB3 to persist in the adult CNS following neonatal infection (17). We speculate that neural stem cells in the adult CNS may be a site of viral persistence, and that CVB3 may undergo active viral replication during stem cell proliferation and division. Conversely, we expect that viral replication and viral protein expression may become substantially reduced within quiescent primary stem cells, as opposed to rapidly proliferating progenitor cells (14). As we have shown, acute CVB3 replication may harm NPSCs in culture, and this injurious effect on stem cell function may occur in vivo as well. In fact, viral RNA by itself may be damaging to normal brain function, as shown for myocytes in culture (39). Also, the expression of viral proteins during CVB3 replication may activate the host immune response which may compromise neural stem cell function.

We evaluated the ability of CVB3 to target neuronal, oligodendrocyte and astrocyte precursor cells through immunofluorescence co-localization studies using antibodies against neuronal class III β-tubulin (immature neurons), NG2/Olig2 (oligodendrocyte precursor markers), myelin basic protein (MBP - mature oligodendrocyte marker), and GFAP (glial/astrocyte precursor marker), and (13) (33) (22). Oligodendrocytes are critical cells in the CNS and provide axons with insulating myelin sheaths. Astrocytes, once considered merely support cells in the brain are now though to play a more active role by affecting the activity of neurons (22). CVB3 appeared to preferentially target nestin+ and NG2+ cells for infection. It is not clear from our
studies which cells might preferentially undergo cpe following infection. However, any reduction in nestin* and NG2* progenitor cells following infection/cpe might alter the percentage of downstream oligodendrocyte precursor cells and potentially impact normal myelination in the developing CNS. Also, well-differentiated cultures expressing high levels of GFAP, β-tubulin, or MBP, and lacking nestin expression, failed to support CVB3 infection as determined by viral protein expression or by viral titers. Of note, differentiation of infected actin-GFP NPSCs resulted in an increase of neuronal class III β-tubulin-expressing cells, as compared to mock-infected controls. However, no statistically significant changes within infected C57 BL/6 NPSCs were observed following their differentiation.

In summary, we propose that CVB3 may target neural stem cells in culture, induce cpe preferentially in progenitor cells as compared to their differentiated counterparts, and may persist in neurosphere cultures replenished with fresh complete NPSC media. Taken together, our results suggest that virus protein expression was robust in undifferentiated neurospheres, yet differentiated cells adjacent to infected neurospheres appeared to be refractory to infection. Future studies will evaluate neural stem cell function and potential genomic alterations in the CVB3 genome within persistently-infected NPSC cultures. Also, actin-GFP NPSCs will assist us with future experiments designed to track previously-infected neural stem cells adoptively transferred within a new host. We will test the ability of NPSCs surviving infection to continue functioning normally, give rise to the three downstream neural cell lineages and migrate correctly within an in vivo environment.
Reference List


Acknowledgements

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**FIGURE LEGENDS**

**Figure 1. Neurospheres grown in culture were highly susceptible to eGFP-CVB3 infection.** NPSCs were isolated from the cortices of one day-old C57 BL/6 mice, cultured to form neurosphere aggregates, and infected with eGFP-CVB3 (moi = 0.1). Infected neurospheres were observed over time by fluorescence microscopy and viral titers were determined by plaque assay. (A) Virus protein expression (green) was readily observed by day 1 PI. (B), (C) An increase in viral protein expression was seen until day 4 PI. (D) Higher magnification of (C) showed the preferential infection of neurosphere aggregates with little to no viral protein expression in adherent cells with stretched morphology. (E) By day 6 PI, virus protein levels were reduced, and (E) signs of cytopathic effect (cpe) were readily observed at a higher magnification (notched white arrows). (G) By day 7 PI, viral protein levels were low, many neurospheres disappeared, and the remaining cells consisted of adherent cells (white arrow). (H) In contrast, neurospheres were readily apparent in mock-infected cultures (white arrow). (I) Viral titers increased over time and reached a maximum at day 3 PI (yellow line with circles). Also, infected NPSCs replenished with complete media every three days supported a carrier state infection with high levels of infectious virus (pink line with squares).

**Figure 2. CVB3 preferentially infected nestin+ and NG2+ cells in neurospheres.** NPSCs were infected with eGFP-CVB3, harvested after day 2 PI, and stained for four cell markers (nestin, neuronal class III β-tubulin, GFAP, and NG2). Structured illumination immunofluorescence microscopy was carried out with a Zeiss Axio Observer with Apotome Imaging System. (A) Both nestin⁺ (white arrows) and nestin⁻ cells (notched blue arrows) were susceptible to infection within neurosphere aggregates. Higher magnification of the field outlined by a hatched box showed both nestin⁺ and nestin⁻ infected cells (white arrows and notched blue arrows, respectively). Also, infected cells expressed cell lineage markers, including β-tubulin,
GFAP, and NG2 cell markers. Higher magnification of the field outlined by a hatched box for each cell lineage marker showed both lineage$^+$ (white arrows) and lineage$^-$ (notched blue arrows) infected cells. (B) The percentage of infected cells expressing each marker at day 1 and day 2 PI was quantified and represented in a table form. A relatively high percentage of nestin$^+$ and NG2$^+$ cells were infected with eGFP-CVB3 at day 1 and day 2 PI. A lower percentage of infection was seen in cells expressing $\beta$-tubulin and GFAP at day 1 and day 2 PI.

Figure 3. Virus replication and cytopathic effects in neurospheres isolated from actin promoter-GFP transgenic mice and infected with dsRED-CVB3. Actin promoter-GFP (actin-GFP) NPSCs were isolated from the cortices of one day-old actin-promoter GFP transgenic mice, cultured to form neurosphere aggregates, and infected with dsRED-CVB3 (moi = 0.1 and 10.0). Infected actin-GFP NPSCs were observed over 7 days by fluorescence microscopy and viral titers were determined by plaque assay. (A-E) actin-GFP NPSCs infected at the lower moi (moi = 0.1) expressed high levels of virus protein in the majority of neurospheres (red; inset images without green signal) as soon as day 3 PI (white arrows). Cytopathic effects were seen at day 5 PI. (F-J) Accelerated virus protein expression (red; inset images without green signal) at day 2 PI (white arrows) and cpe (at day 3 PI) were seen in actin-GFP NPSCs infected with the higher moi (moi = 10.0). (K), (M) Higher magnification of (G), and a single channel image (dsRED), revealed the relatively high level of virus protein expression and the presence of dying cells (white arrow) in day 2 PI actin-GFP NPSCs infected at the higher moi. (L) Similarly, higher magnification of (C) showed dsRED expression and the presence of dying cells (white arrow) in day 3 PI actin-GFP NPSCs infected at the lower moi. (N) No red signal or cpe was observed in mock-infected actin-GFP NPSCs. (O) Viral titers were determined for actin-GFP NPSCs infected at a low (light pink line with squares) or high moi (dark pink with circles).
Figure 4. Quantification of virus-induced cell death over time in neurospheres following infection with two recombinant coxsackieviruses. C57 BL/6 NPSCs or actin-GFP NPSCs were mock-infected or infected with recombinant CVBs (eGFP-CVB3 and dsRED-CVB3, respectively) at two multiplicities of infection (moi), observed by fluorescence microscopy, and stained for trypan blue at day 3 PI. (A-C) Greater levels of virus protein (green) and accelerated cpe was observed in NPSCs infected with eGFP-CVB3 at higher moi (white arrow). (D-F) Similarly, greater levels of virus protein (red) and accelerated cpe was observed in actin-GFP NPSCs infected with dsRED-CVB3 a higher moi (white arrow). (G), (H) The percentage of dead (trypan blue*) cells was determined in infected C57 BL/6 and actin-GFP NPSCs using a hemacytometer. A greater percentage of dead cells was observed at each time point in both NPSC cultures infected with the higher moi.

Figure 5. Differentiation of NPSCs following treatment with fetal bovine serum. C57 BL/6 NPSCs were evaluated for nestin and β-tubulin expression before and after FBS treatment. (A) NPSCs were grown in complete NPSC media, or alternatively, were differentiated for 5 days in the presence of FBS. NPSCs or differentiated NPSCs were fixed with 2% paraformaldehyde and stained for nestin (red), or co-stained for both nestin (green) and β-tubulin (red). Cells were counterstained with DAPI (nuclear dye). (B) NPSCs differentiated for 5 or 16 days in the presence of FBS were placed into complete NPSC media in the absence of FBS (NPSC Media) for an additional 3 or 7 days. Partial “de-differentiation” was observed as soon as day 3 post-treatment for NPSCs differentiated for 5 days with complete “de-differentiation” at day 7 post-treatment. Also, partial “de-differentiation” was observed by day 7 post-treatment for NPSCs differentiated for 16 days. (C) NPSCs were differentiated for 5 or 16 days and placed in NPSC media or in the presence of FBS (Diff. Media) for 3 days, fixed with 2% paraformaldehyde, and immunostained for nestin, GFAP, neuronal class III β-tubulin (β-tubulin), or myelin basic protein.
5 Diff. and 16 Day Diff. cells expressed high levels of nestin and reduced levels of GFAP and β-tubulin in the presence of complete NPSC media, suggesting their “de-differentiation” in these cultures. 16 Day Diff. cells in Diff Media expressed little or no nestin (white arrows), suggesting their differentiated status.

**Figure 6. Higher levels of viral protein expression and cpe were observed in NPSCs, as compared to differentiated NPSCs.** NPSCs or differentiated NPSCs (5 Day Diff. or 16 Day Diff.) were infected with eGFP-CVB3 (moi = 0.1) and followed for virus protein expression for 10 days PI. Following infection, NPSCs or differentiated NPSCs were cultured in complete NPSC media lacking FBS (NPSC Media, pink arrow). Alternatively, differentiated NPSCs infected with virus were cultured in media containing FBS (Diff. Media, yellow arrow). The greatest to least amount of virus protein expression and cpe was observed in the following order from left to right: NPSCs + NPSC Media > 5 Day Diff. + NPSC Media > 16 Day Diff. + NPSC Media > 5 Day Diff. + Diff. Media > 16 Day Diff. + Diff. Media. Despite the relatively high level of cpe, surviving neurospheres continued to be observed in infected NPSCs + NPSC Media, and in infected 5 Day Diff. + NPSC Media (white arrows).

**Figure 7. Higher levels of viral titers observed in NPSCs, as compared to differentiated NPSCs.** NPSCs or differentiated NPSCs (5 Day Diff. or 16 Day Diff.) were infected with eGFP-CVB3 (moi = 01) and observed by fluorescence microscopy over time. Also, viral titers were evaluated in these cultures up to 7 days PI. Following infection, NPSCs or differentiated NPSCs were cultured in complete NPSC media lacking FBS (NPSC Media). Alternatively, differentiated NPSCs infected with virus were cultured in media containing FBS (Diff. Media). (A) Infected cells at day 3 PI with extended axonal processes were readily apparent in 5 Day Diff. + NPSC Media (white arrows). (B), (C), (D) Higher magnification of (A) showed the contact of infected cells with axonal processes to adjacent uninfected cells. (E), (F) Cellular “blebbing” was seen in
many differentiated NPSCs following infection, including 5 Day Diff. + NPSC Media at day 2 PI (notched light blue arrows), and in 5 Day Diff + Diff. Media at day 7 PI (notched light blue arrows). Neurospheres surviving infection in NPSCs + NPSC Media cultures were observed at (G) day 10 PI and (H) day 37 PI. (I), (J) Detectable levels of virus protein expression were observed in many surviving neurospheres at day 37 PI (notched light blue arrows). (K) Viral titers were determined for NPSCs and compared to 5 Day Diff. NPSCs cultured in Diff. Media or NPSC Media. 5 Day Diff. + Diff. Media cultures produced the least amount of infectious virus over 7 days PI. (L) Viral titers were determined for NPSCs and compared to 16 Day Diff. NPSCs cultured in Diff. Media or NPSC Media. 16 Day Diff. + Diff. Media cultures failed to produce detectable levels of infectious virus for up to 3 days PI.

**Figure 8. Reduction in CVB3 replication and viral protein expression in highly differentiated NPSCs expressing neural differentiation markers and lacking nestin expression.** C57 BL/6 NPSCs were differentiated in the presence of FBS for 5 or 16 days and infected with eGFP-CVB3 (moi = 0.1 or 1.0). After infection, differentiated NPSCs were cultured in NPSC Media or Diff. Media. Also, viral titers were evaluated in these cultures up to 14 days PI. (A) After day 2, 3, or 14 PI, cultures were stained for nestin and three downstream cell lineage markers (GFAP, β-tubulin, and MBP). Moderate to high levels of all three cell lineage markers were observed in all cultures. Single channel black and white images for insets show the expression level for each cell lineage marker. Also, little to no nestin expression was observed in 16 Day Diff. + Diff. Media cultures, indicating well-defined differentiation of these cells. The highest level of viral protein expression was observed in 5 Day Diff. + NPSC Media. Detectable levels of viral protein expression were seen in 5 Day Diff. + Diff. Media. Higher magnification of hatched boxes showed co-localization of viral protein expression in cells moderate levels of diffuse nestin and in some β-tubulin* cells (white arrows). In contrast, a lack...
of co-localization was observed for GFAP and MBP staining (notched blue arrows). Little to no viral protein expression was observed in 16 Day Diff. + Diff. Media. (B) 5 Day Diff. + Diff. Media cultures supported low to moderate levels of viral replication depending upon moi utilized at the single time point analyzed. (C) In contrast 16 Day Diff. + Diff. Media cultures supported lower levels of viral replication at either moi utilized, and these levels dropped to below detection limits at day 14 PI.

**Figure 9. CVB3 infection and alteration of the NPSC differentiation pathway.** C57 BL/6 NPSCs were infected with eGFP-CVB3 (moi = 0.1). In parallel, actin-GFP NPSCs were infected with dsRED-CVB3 (moi = 0.1). After day 2 PI, infected NPSCs were differentiated in the presence of FBS for three days and harvested for immunofluorescence staining. Infected NPSCs were stained for three downstream cell lineage markers (GFAP, β-tubulin, and Olig2). For C57 BL/6 NPSCs, the relative fluorescence of each marker following infection was calculated and normalized to DAPI signal using ImageJ program. For actin-GFP NPSCs, the relative fluorescence of each marker following infection was calculated and normalized to GFP signal using ImageJ software (inset images shown for single blue channel).
Figure 1. Neurospheres grown in culture were highly susceptible to eGFP-CVB3 infection.
Figure 2. CVB3 preferentially infected nestin⁺ and NG2⁺ cells in neurospheres.

A

Day 2 Post-Infection
Zeiss Axio Observer with Apotome Imaging System

B

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pos. Cells</th>
<th>Neg. Cells</th>
<th>Total</th>
<th>% Positive</th>
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<td>Nestin</td>
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<td>5</td>
<td>15</td>
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<td>4</td>
<td>40</td>
<td>44</td>
<td>9.1</td>
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<tr>
<td>GFAP</td>
<td>10</td>
<td>86</td>
<td>96</td>
<td>10.4</td>
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<tr>
<td>NG2</td>
<td>12</td>
<td>18</td>
<td>30</td>
<td>40.0</td>
</tr>
<tr>
<td>Day 1 PI</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Nestin</td>
<td>36</td>
<td>12</td>
<td>48</td>
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<tr>
<td>β-tubulin</td>
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<td>47</td>
<td>14.9</td>
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<tr>
<td>GFAP</td>
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<td>52</td>
<td>61</td>
<td>14.8</td>
</tr>
<tr>
<td>NG2</td>
<td>31</td>
<td>13</td>
<td>44</td>
<td>70.5</td>
</tr>
</tbody>
</table>

Day 2 PI
Figure 3. Virus replication and cytopathic effects in neurospheres isolated from actin promoter-GFP transgenic mice and infected with dsRED-CVB3.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Actin-GFP Neurospheres</th>
<th>Hoffmann M.C.</th>
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<tr>
<td><strong>Day 1</strong></td>
<td><strong>Day 2</strong></td>
<td><strong>Day 3</strong></td>
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<tr>
<td>MOI = 0.1</td>
<td>A</td>
<td>B</td>
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<td></td>
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</tr>
<tr>
<td><strong>Day 5</strong></td>
<td><strong>Day 7</strong></td>
<td></td>
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<tr>
<td>MOI = 10.0</td>
<td>F</td>
<td>G</td>
</tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>L</td>
</tr>
</tbody>
</table>

Additional plot showing viral titre (PFU/ml) over days post-infection.
Figure 4. Quantification of virus-induced cell death over time in neurospheres following infection with two recombinant coxsackieviruses.
Figure 5. Differentiation of NPSCs following treatment with fetal bovine serum
Figure 6. Higher levels of viral protein expression and cpe were observed in NPSCs, as compared to differentiated NPSCs.
Figure 7. Higher levels of viral titers observed in NPSCs, as compared to differentiated NPSCs.
Figure 8. Reduction in CVB3 replication and viral protein expression in highly differentiated NPSCs expressing neural differentiation markers and lacking nestin expression.

A

NPSCs

\[ \text{Diff Media} \]

\[ \text{Differentiated with FBS} \]

\[ \text{MOI} = 0.1 \]

\[ \text{MOI} = 1.0 \]

\[ \text{MOI} = 1.0 \]

5 Day Diff.

Virus Nestin DAPI

18 Day Diff.

Virus Nestin DAPI

5 Day Diff.

Virus GFAP DAPI

Day 2 PI

Day 3 PI

Day 14 PI

Virus \( \beta \)-tubulin DAPI

Virus MBP DAPI


B

NPSCs Differentiated For 5 Days + Diff Media

\[ \text{DS Diff (MOI=0.1)} \]

\[ \text{DS Diff (MOI=1.0)} \]

Viral Titters (pfu/ml)

1.0 \times 10^{10}

1.0 \times 10^{9}

1.0 \times 10^{8}

1.0 \times 10^{7}

1.0 \times 10^{6}

1.0 \times 10^{5}

1.0 \times 10^{4}

1.0 \times 10^{3}

1.0 \times 10^{2}

Days Post - Infection

C

NPSCs Differentiated For 16 Days + Diff Media

\[ \text{D16 Diff (MOI=0.1)} \]

\[ \text{D16 Diff (MOI=1.0)} \]

Viral Titters (pfu/ml)

1.0 \times 10^{10}

1.0 \times 10^{9}

1.0 \times 10^{8}

1.0 \times 10^{7}

1.0 \times 10^{6}

1.0 \times 10^{5}

1.0 \times 10^{4}

1.0 \times 10^{3}

1.0 \times 10^{2}

Days Post - Infection

* Below detection limits
Figure 9. CVB3 infection and alteration of the NPSC differentiation pathway.