



# Differentiated Human SH-SY5Y Cells Provide a Reductionist Model of Herpes Simplex Virus 1 Neurotropism

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**ABSTRACT** Neuron-virus interactions that occur during herpes simplex virus (HSV) infection are not fully understood. Neurons are the site of lifelong latency and are a crucial target for long-term suppressive therapy or viral clearance. A reproducible neuronal model of human origin would facilitate studies of HSV and other neurotropic viruses. Current neuronal models in the herpesvirus field vary widely and have caveats, including incomplete differentiation, nonhuman origins, or the use of dividing cells that have neuropotential but lack neuronal morphology. In this study, we used a robust approach to differentiate human SH-SY5Y neuroblastoma cells over 2.5 weeks, producing a uniform population of mature human neuronal cells. We demonstrate that terminally differentiated SH-SY5Y cells have neuronal morphology and express proteins with subcellular localization indicative of mature neurons. These neuronal cells are able to support a productive HSV-1 infection, with kinetics and overall titers similar to those seen in undifferentiated SH-SY5Y cells and the related SK-N-SH cell line. However, terminally differentiated, neuronal SH-SY5Y cells release significantly less extracellular HSV-1 by 24 h postinfection (hpi), suggesting a unique neuronal response to viral infection. With this model, we are able to distinguish differences in neuronal spread between two strains of HSV-1. We also show expression of the antiviral protein cyclic GMP-AMP synthase (cGAS) in neuronal SH-SY5Y cells, which is the first demonstration of the presence of this protein in non-epithelial cells. These data provide a model for studying neuron-virus interactions at the single-cell level as well as via bulk biochemistry and will be advantageous for the study of neurotropic viruses *in vitro*.

**IMPORTANCE** Herpes simplex virus (HSV) affects millions of people worldwide, causing painful oral and genital lesions, in addition to a multitude of more severe symptoms such as eye disease, neonatal infection, and, in rare cases, encephalitis. Presently, there is no cure available to treat those infected or prevent future transmission. Due to the ability of HSV to cause a persistent, lifelong infection in the peripheral nervous system, the virus remains within the host for life. To better understand the basis of virus-neuron interactions that allow HSV to persist within the host peripheral nervous system, improved neuronal models are required. Here we describe a cost-effective and scalable human neuronal model system that can be used to study many neurotropic viruses, such as HSV, Zika virus, dengue virus, and rabies virus.

**KEYWORDS** neuron, virus, HSV-1, differentiation, infection, SH-SY5Y, SK-N-SH, cGAS, herpes simplex virus

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus in the *Alphaherpesvirinae* subfamily of *Herpesviridae*. The virus establishes primary infection in humans through entry at skin and mucosal sites. The orofacial region is the most common site of initial infection, but genital tract infections are also prevalent (1). It is estimated

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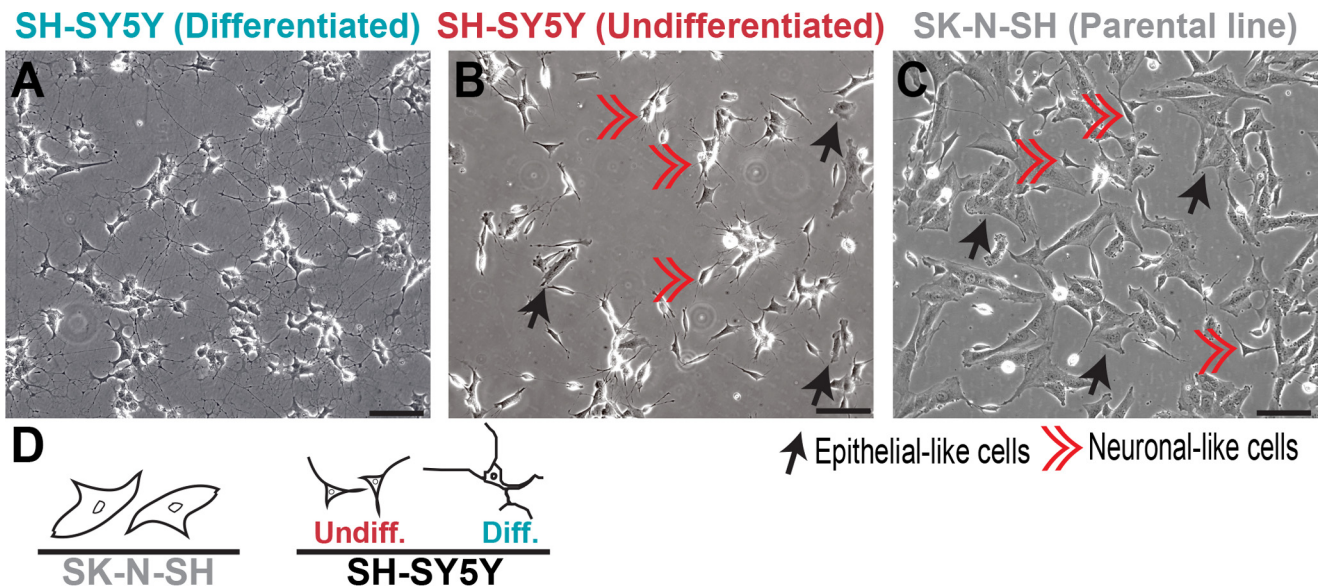
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that approximately 67% of the world's population is infected orally with HSV-1, while approximately 19% are infected genitally with either HSV-1 or -2 (2, 3). In addition to replicating at the peripheral epithelium, HSV is able to establish latent infections in sensory and autonomic ganglia via retrograde axonal transport into the peripheral nervous system. Once latent, the virus can reactivate at any time in response to stimuli such as physical or emotional stress or compromised immunity. Cell- or tissue-specific factors play a role in the establishment of a lytic versus latent infection, as evidenced by the ability of the virus to induce latency in neurons, while infection in nonneuronal cells remains productive (1). However, viral replication does occur in human neurons. Evidence suggests that neurons can undergo productive infection prior to establishing a quiescent, latent state, and neurons also actively produce virus during reactivation from latency (4, 5). At present, there is no vaccine available to prevent HSV infection. Current antiviral therapies treat the active, replicating stage of the virus, leaving the latent phase unaffected. Development of new antivirals that target latent virus in neurons will be key to ending lifelong infections. A reproducible *in vitro* model of mature, homogeneous, human neurons will facilitate the search for new antivirals that target HSV latency and reactivation (6).

Immune molecules play a key role during HSV-1 infection in both the epithelium and the peripheral nervous system. The severity of HSV-1 disease that an individual experiences is thought to depend on a combination of viral virulence factors, local environmental stimuli (such as UV exposure), and the ability of the host's immune system to combat the virus (7–11). Pathogen recognition to initiate the host immune response occurs via many different pathways and mechanisms (12–14). Cyclic GMP-AMP synthase (cGAS) and interferon gamma (IFN- $\gamma$ )-inducible protein 16 (IFI16) are two viral DNA sensors shown to be important for detecting HSV-1 in epithelial cells and eliciting a downstream immune response through interferon regulatory factor 3 (IRF-3) (15–21). Recent work with epithelial cells suggests that IFI16 is required for direct detection of and binding to HSV-1 DNA in the nucleus, while cGAS acts to stabilize this interaction (20). However, it is unknown whether this same mechanism exists in neurons. It is known that neurons express Toll-like receptor 3 (TLR-3) in both the brain and peripheral nerves *in vivo* (22–24). It has also been shown that TLR-3 can play diverse antiviral roles in the host and that its function may be dependent upon other immune factors and/or the specificity of the invading pathogen (25, 26). Neurons express major histocompatibility complex (MHC) class I molecules *in vivo* (27, 28), and expression of these molecules is increased after infection with murine hepatitis virus or HSV (29, 30). Since neurons *in vivo* express DNA sensors that serve as detectors of viral infection, it is important to determine if *in vitro* neuronal models reflect this capability as well.

Current *in vitro* research on HSV-1 infection of neurons is often performed using murine neurons, totipotent stem cells, or undifferentiated neuroblastoma cells (31–34). Although useful, these systems have caveats, such as incomplete neuronal differentiation, ongoing cell division, variability in neuronal phenotypes, nonhuman origins, and/or unstable karyotypes (31, 35, 36). These have negative impacts on experimental reproducibility. Furthermore, the use of rodent instead of human neurons introduces additional caveats, such as differences in the outcome of transcription factor cascades in mice versus humans (37–39). Herpesviruses are also highly host specific, having coevolved with their hosts over millions of years (40). HSV-1 penetration into the central nervous system (CNS) is uncommon in human adults, as is severe neurologic disease such as encephalitis (41, 42). Murine model systems reach this encephalitic outcome more readily than human HSV-1 infections (8, 43, 44). These findings highlight the importance of using a system that reflects the attributes of human neurons, and the need to corroborate prior results from murine models in a human neuronal environment.

Prior data have established the ability of the chromosomally stable human SH-SY5Y neuroblastoma cell line to be differentiated into mature neuronal cells (45–48). SH-SY5Y neuroblastoma cells are a subclone derived from the parent cell line SK-N-SH (47). Undifferentiated, dividing SH-SY5Y cells become terminally differentiated over the



**FIG 1** Terminally differentiated neuronal SH-SY5Y cells are morphologically distinct from undifferentiated SH-SY5Y cells and SK-N-SH cells. (A) Terminally differentiated SH-SY5Y cells appear neuronal after 2.5 weeks of differentiation. At 2 days postplating, mixed cell morphologies are visible in undifferentiated SH-SY5Y cells (B) and in the parent cell line SK-N-SH (C). Black arrows indicate epithelial-like cells, and red arrowheads indicate neuronal-like cells. (D) Traced outlines of representative cells, approximately 1.5 times their actual size. Scale bars represent 100  $\mu\text{m}$ .

course of 2.5 weeks. Terminally differentiated in this case is defined as a state in which the cells are no longer dividing, are neurotrophin dependent, and are committed to a neuronal phenotype. During this transition, epithelial-like cells are removed through a combination of serum starvation and the addition of retinoic acid (RA). Serum starvation has been shown to induce apoptosis in epithelial cells (49, 50), while RA activates tyrosine receptor kinase B (Trk) in neuronal cells, leading to survival of only the neurotrophin-dependent cells (45, 51, 52). As neurotrophic factors are added and the cultures are replated onto extracellular matrix (ECM), the cells begin to grow and develop neuritic processes, ultimately forming an extensive meshwork of neurites (53). Eventually, there are few to no epithelial cells remaining, resulting in a homogeneous population of cells that resemble mature human neurons.

Here we characterize the use of terminally differentiated SH-SY5Y neuronal cells (53) as a model for studying HSV-1–neuron interactions during the course of infection. This system provides a model with a natural host-virus species pairing which is amenable to both single-cell imaging and bulk biochemical approaches. We demonstrate that terminally differentiated SH-SY5Y cells express neuronal proteins with a localization that is indicative of mature neurons. We show that in a single round of replication, HSV-1 strain McKrae reaches overall titers in terminally differentiated SH-SY5Y neuronal cells similar to those in the mixed population of parental SK-N-SH cells and the undifferentiated SH-SY5Y cell line. However, the neuronal SH-SY5Y cells release significantly less extracellular virus by 24 h postinfection (hpi). In an assay focused on cell-to-cell spread in SH-SY5Y neuronal cells, we were able to detect differences in neuronal spread between the HSV-1 strains McKrae and KOS. These data suggest that this neuronal model may provide an opportunity to quantify the phenotypes of neurotropic viruses *in vitro*. We demonstrate for the first time that these neuronal cells express the host antiviral factor cGAS, which is known to elicit a downstream innate immune response through the detection of viral pathogens.

## RESULTS

### Terminally differentiated SH-SY5Y cells exhibit mature neuronal morphology.

All three cell populations examined in this study (Fig. 1) have been used previously to study neuronal aspects of host-virus interactions (Table 1). However, SK-N-SH cells and

**TABLE 1** Use of undifferentiated SH-SY5Y and SK-N-SH cells, in contrast to terminally differentiated SH-SY5Y neuronal cells, in studies of neurotropic viruses

Virus type	Virus(es) studied using the indicated cell type (reference[s])		
	SK-N-SH cell line	Undifferentiated SH-SY5Y cells	Differentiated SH-SY5Y cells <sup>a</sup>
DNA viruses	HSV-1 (103, 106–116), <sup>b</sup> JC virus (117)	Adenovirus (118), Epstein-Barr virus (119), HSV-1 (106, 120–125), Kaposi's sarcoma herpesvirus (119), JC virus (117), mouse gammaherpesvirus 68 (126)	Bovine herpesvirus (127), HSV-1 (45, 48, 121), varicella-zoster virus (46, 128)
RNA viruses	Borna disease virus (129), dengue virus (130), enterovirus 71 (131), Japanese encephalitis virus (132, 133), measles virus (134–136), Menangle virus (153), rabies virus (111, 154, 155), Sindbis virus (156), tick-borne encephalitis virus (157), Tioman virus (153), Tofla virus (137)	Dengue virus (138), enterovirus 71 (139, 140), HIV (141, 142), human endogenous retrovirus (143), Japanese encephalitis virus (144, 145), mumps virus (158)  Newcastle disease virus (159), poliovirus (55), porcine reproductive and respiratory syndrome virus (160), Puumala virus (161), rabies virus (154), West Nile virus (54), western equine encephalitis virus (56), vesicular stomatitis virus (162), Zika virus (146)	Chikungunya virus (147), enterovirus 71 (148), HIV (149–152), human T-cell lymphotropic virus (163)

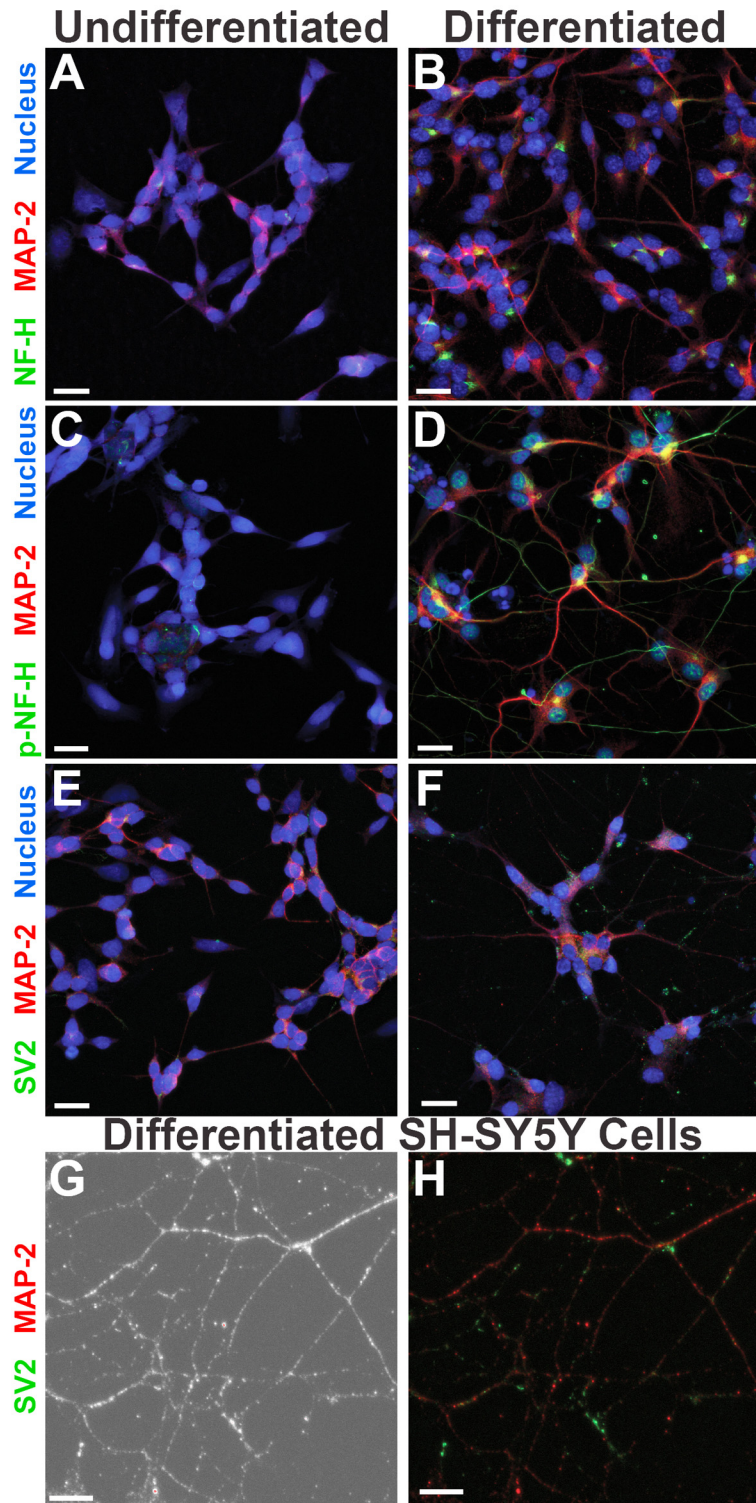
<sup>a</sup>Includes both short (5 to 7 days) and long differentiation (>10 days) protocols.

<sup>b</sup>In the studies described in references 115 and 116, SK-N-SH cells were differentiated with retinoic acid for 7 days.

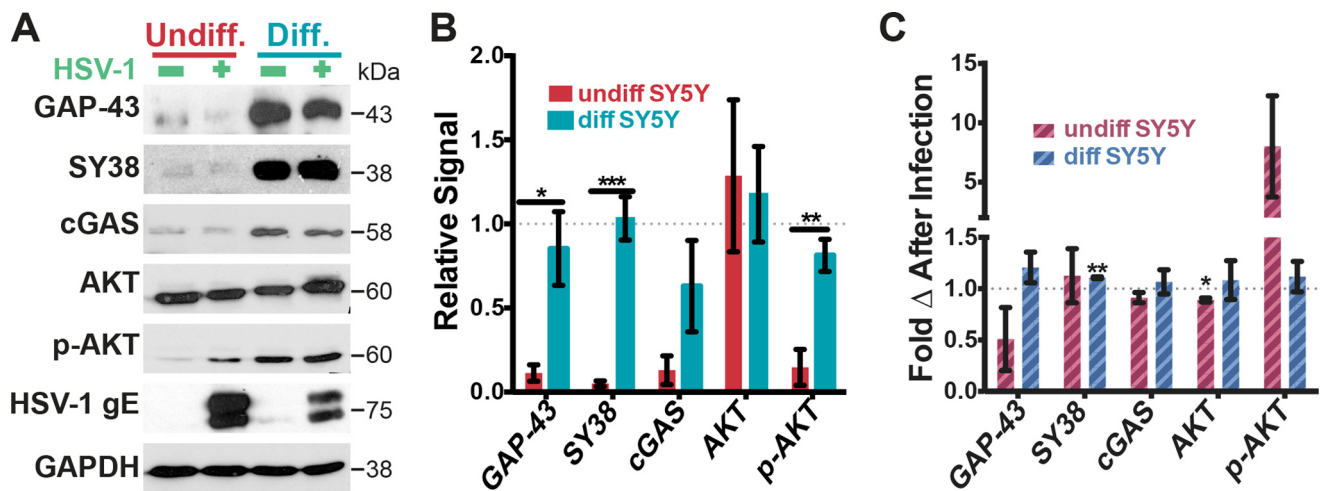
undifferentiated SH-SY5Y cells experience ongoing cell division and contain mixed populations of epithelial-like and neuronal-like cells (47). This raised the possibility that using these cells in an undifferentiated state would reflect epithelial or mixed cell population responses to infection, rather than the intended focus on neuronal responses. Terminally differentiated SH-SY5Y cells provide a homogeneous population of human neuronal cells that can be used to study neuron-virus interactions for a variety of neurotropic viruses that impact public health (54–57). We investigated whether differentiated SH-SY5Y neuronal cells served as a suitable platform to study human host-virus interactions in a species-matched manner.

Differentiation of SH-SY5Y cells for 2.5 weeks, with removal of serum and addition of neurotrophic factors, yielded neuronal cells (see Materials and Methods for details). Morphologically, these differentiated neuronal cells were distinct from both the parental SK-N-SH cell line and from the undifferentiated SH-SY5Y progenitor cells (Fig. 1). Terminally differentiated neuronal cells had a smaller soma with extensive neurite projections (Fig. 1A), whereas the parental SK-N-SH cells had an epithelial appearance with a larger soma (Fig. 1C). Undifferentiated SH-SY5Y cells were a heterogeneous population of epithelial-like cells that resembled those of the SK-N-SH parent line, and neuronal-like cells with short neurites (Fig. 1B).

Previously published data have demonstrated that differentiated human neurons have enhanced expression of specific neuronal proteins compared to that of nonneuronal cells, including neurofilament components, synaptic vesicle proteins, and growth-associated proteins, such as growth-associated protein 43 (GAP-43) (45, 46, 58, 59). We used immunofluorescence (IF) to ascertain whether terminally differentiated SH-SY5Y cells express localized neuronal markers similar to those found in mature neurons. Following 2.5 weeks of differentiation, both the undifferentiated (Fig. 2A) and differentiated (Fig. 2B) SH-SY5Y cells expressed microtubule-associated protein 2 (MAP-2), with more extensive localized staining visible in the differentiated cells due to their expansive neurite network. Both undifferentiated and differentiated SH-SY5Y cells expressed the unphosphorylated form of neurofilament heavy chain (NF-H) protein, which localized in the perinuclear space (Fig. 2A and B). Phosphorylated NF-H (p-NF-H) is a marker of axons in mature neurons (60). We found that this protein was expressed only in the differentiated SH-SY5Y cells, where it localized in axonal projections (Fig. 2C and D). Synaptic vesicle protein 2 (SV2) specifically localizes at synapses in neurons (61), and only the neuronal SH-SY5Y cells appeared to have concentrated puncta of SV2 present in their dense network of neurites (Fig. 2E to H). Based on morphology and immunofluorescence data, terminally differentiated SH-SY5Y cells appeared neuronal and fully polarized.



**FIG 2** Terminally differentiated SH-SY5Y neuronal cells show distinct localization of cytoskeletal and synaptic markers. Differentiation of SH-SY5Y cells for 2.5 weeks yields morphologically distinct localization of neuronal proteins (B, D, and F), in contrast to that seen in undifferentiated SH-SY5Y cells (A, C, and E). Higher magnification of differentiated SH-SY5Y neurites shown in phase-contrast (G) reveals further details of microtubule and synaptic vesicle staining (H). The 2.5-week differentiation process includes the addition of RA, neurotrophic factors, and ECM proteins. MAP-2, microtubule associated protein 2; NF-H, unphosphorylated neurofilament heavy chain; p-NF-H, phosphorylated neurofilament heavy chain; SV2, synaptic vesicle protein 2; Nucleus, nuclear/DNA stain. Images were taken with an Olympus FV10i confocal microscope at a magnification of  $\times 60$ , and the scale bars represent  $20 \mu\text{m}$ . Images in panels G and H were taken with an additional  $2.6\times$  digital zoom, and the scale bars represent  $10 \mu\text{m}$ .



**FIG 3** Neuronal differentiation significantly impacts expression of GAP-43, SY38, and p-AKT. (A) Proteins involved in neuronal differentiation, growth, and signaling are expressed at high levels in terminally differentiated SH-SY5Y neuronal cells. The neuronal growth-associated protein GAP-43 and synaptic vesicle marker SY38 are expressed at significantly higher levels in differentiated SH-SY5Y neuronal cells than in the undifferentiated progenitor cells. The cytoplasmic DNA sensor cGAS, as part of the intrinsic immune response, is expressed in both the undifferentiated and neuronal SH-SY5Y cells. While AKT levels appear equal in undifferentiated and neuronal SH-SY5Y cells, p-AKT levels are significantly higher in differentiated neuronal cells. HSV-1 glycoprotein E (gE) serves as a positive control for viral infection. GAPDH serves as a loading control. Images are representative of three biological replicates. Twenty micrograms of protein was loaded for each cell population. (B) Quantification of the effects of differentiation on protein expression levels in uninfected SH-SY5Y cells that are either undifferentiated (red) or neuronally differentiated (blue). The signal of each protein target was normalized and expressed relative to GAPDH loading levels. p-AKT signal was normalized to AKT. Multiple *t* tests were performed with alpha at 5.0%. \*,  $P = 0.03$ ; \*\*,  $P = 0.01$ ; \*\*\*,  $P = 0.002$ . (C) HSV-1 infection has a significant impact on SY38 and AKT protein levels in SH-SY5Y cells. With the exception of p-AKT induction in undifferentiated SH-SY5Y cells and SY38 expression in differentiated SH-SY5Y neuronal cells, most protein levels are unchanged following HSV-1 infection. Results are presented as fold change of expression following infection. One-sample *t* tests were performed on each protein target against a hypothetical uninfected value of 1. \*,  $P = 0.03$ ; \*\*,  $P = 0.007$ . See the text for protein descriptions and Materials and Methods for antibody details.

### Neuronal differentiation significantly impacts expression of GAP-43, SY38, and phospho-AKT (p-AKT).

After we determined that the differentiated SH-SY5Y cells had specific localization and expression of neuronal markers, we next investigated protein levels by Western blotting (WB). We compared protein levels in the undifferentiated and terminally differentiated neuronal states, as well as in the presence of HSV-1 infection. We examined total cell lysates from uninfected cells versus those from cells infected at a multiplicity of infection (MOI) of 10 with HSV-1 McKrae and harvested at 6 hpi. We found that differentiated SH-SY5Y cells had high expression levels of the neuronal proteins GAP-43 and synaptic vesicle protein 38 (SY38) (Fig. 3A and B), corroborating the IF data. GAP-43 phosphoprotein is expressed in neuronal growth cones and aids in the extension and outgrowth of neurites in developing neurons (62). We found that GAP-43 expression was significantly higher in the differentiated SH-SY5Y neuronal cells than in the undifferentiated SH-SY5Y cells. However, no significant changes in the expression of this neuronal protein occurred following HSV-1 infection (Fig. 3C). Synaptic vesicle protein 38, also known as synaptophysin, is an integral membrane glycoprotein expressed in presynaptic vesicles of neurons (63). Synaptophysin was expressed at significantly higher levels in the differentiated SH-SY5Y neuronal cells than in undifferentiated SH-SY5Y cells (Fig. 3A and B). SY38 expression in differentiated SH-SY5Y neuronal cells also appeared to significantly increase during HSV-1 infection (Fig. 3C). Taken together, these data demonstrated that terminally differentiated SH-SY5Y cells have high expression of neuronal proteins and that undifferentiated SH-SY5Y cells do not reflect this aspect of neurobiology.

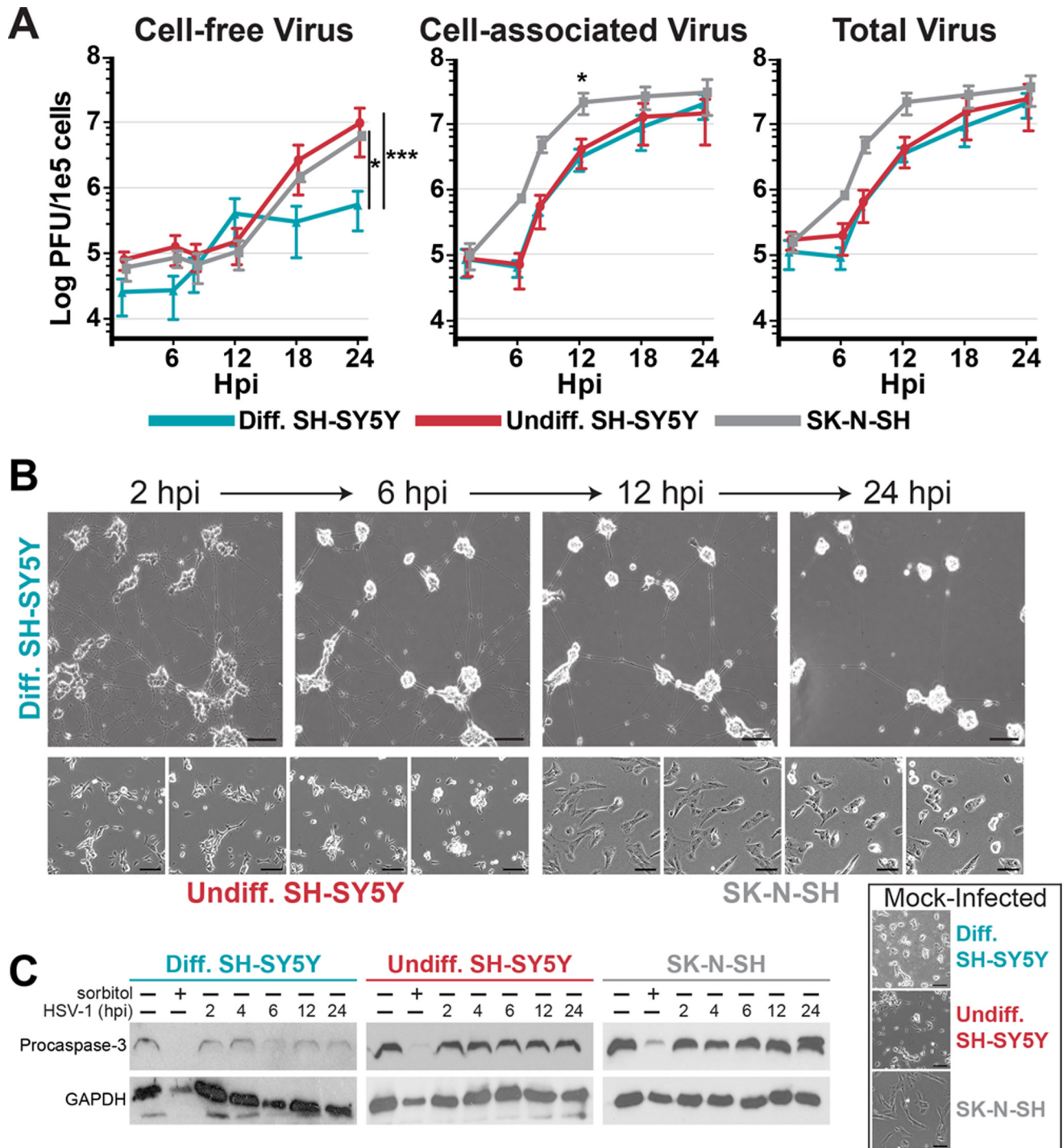
To investigate the response of these neuronal cells to HSV-1 infection, we measured the levels of several antiviral and stress-associated proteins before and after infection. Based on prior data (20, 65), we hypothesized that viral infection would induce a stress response within cells, and that this might alter the expression profile of these proteins following infection. We found that protein kinase B (AKT) expression levels remained similar between differentiation states and that viral infection appeared to significantly

affect AKT expression only in undifferentiated SH-SY5Y cells (Fig. 3). Phosphorylation and activation of AKT (to form phospho-AKT) occurred in both cell types during infection. However, when the cells were not infected, phospho-Akt (p-AKT) was apparent only in terminally differentiated SH-SY5Y neuronal cells (Fig. 3). We also examined the expression levels of the antiviral protein cGAS, which has been implicated in detecting and binding to HSV-1 viral DNA and eliciting a downstream immune response (20). This antiviral protein is also involved in sensing many other viral pathogens (66). We found that cGAS expression levels were higher in the differentiated SH-SY5Y neuronal cells than in the undifferentiated progenitor cells. cGAS expression did not appear to be affected by HSV-1 at 6 hpi (Fig. 3).

**Neuronal SH-SY5Y cells release less extracellular virus during lytic HSV-1 infection.** Next, we wanted to determine whether a commonly used strain of HSV-1 replicated differently in the neuronal cells than in the progenitor cells during a single round of replication. Using the HSV-1 strain McKrae (67–70), we measured the progression of productive HSV-1 infection in this neuronal model compared to the epithelial progenitor cells included in Fig. 1. We performed high-MOI (single-step) growth curves in parental SK-N-SH cells, undifferentiated SH-SY5Y cells, and terminally differentiated SH-SY5Y neuronal cells. Each cell type was infected with HSV-1 McKrae at an MOI of 10, and we collected cell-associated virus and released-virus (cell-free) samples at 1, 6, 8, 12, 18, and 24 hpi. Quantification of virus production suggested that all three cell types had similar viral kinetics and overall titers during a single round of replication (Fig. 4A). However, virion production in SK-N-SH cells appeared to peak earlier (Fig. 4A, 1 to 6 hpi). In addition, terminally differentiated SH-SY5Y neuronal cells released significantly less extracellular virus than the mixed cell populations by 24 hpi (Fig. 4A). These data demonstrated that neuronal SH-SY5Y cells were fully replication competent for HSV-1 and suggested the potential for neuronal phenotypes distinct from those observed in epithelial-like cells.

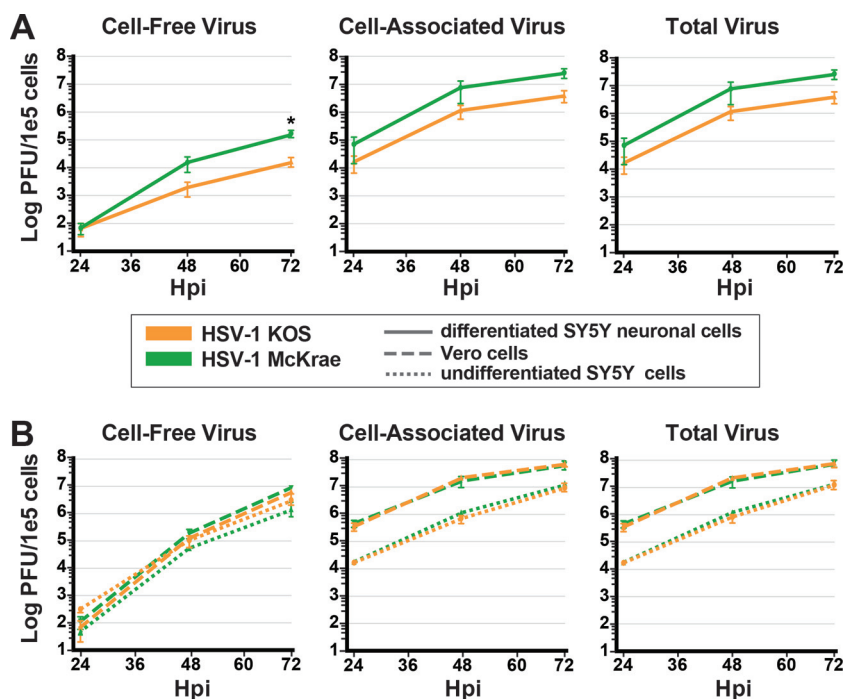
Phase-contrast images of all three cell types infected at an MOI of 10 with HSV-1 McKrae demonstrated cytopathic effects (CPE) over time (Fig. 4B; see also Movie S1 in the supplemental material). Morphological changes in the neuronal cells were identified as early as 6 hpi, including retraction of neurites and loss of substrate attachment. Alterations in the epithelial progenitor cells were not visible until approximately 12 hpi. To investigate whether these cells undergo cell death via caspase-mediated apoptosis, inactive procaspase 3 cleavage was assessed at 2, 4, 6, 12, and 24 hpi (Fig. 4C). Decreased procaspase 3 expression was evident by 6 hpi in the neuronal cells, while procaspase 3 levels did not appear to decrease by 24 h in the undifferentiated SH-SY5Y cells or SK-N-SH progenitor cells. These data suggested that caspase 3 was active early in viral infection of the neuronal cells. These data further supported the morphological changes observed following viral infection over time in the differentiated SH-SY5Y neuronal cells.

**Neuronal SH-SY5Y cells reveal distinct phenotypes of cell-to-cell spread for different strains of HSV-1.** We next determined whether we could distinguish between two frequently studied strains of HSV-1 based upon their ability to replicate and spread from cell to cell in this *in vitro* neuronal model. For this low-MOI (0.01) experiment, we infected differentiated SH-SY5Y neuronal cells with either HSV-1 McKrae or HSV-1 KOS in the presence of 0.1% pooled human serum (see Movie S2). The yield of HSV-1 McKrae infection was approximately 1 log higher than for HSV-1 KOS at 72 hpi (Fig. 5A). Morphologically, the differentiated SH-SY5Y neuronal cells appear to undergo CPE-induced cell death at similar rates over the course of 72 h with both virus strains (Movie S2). In contrast, performing this experiment with both undifferentiated SH-SY5Y cells and Vero cells revealed no difference in the rate of replication between HSV-1 McKrae and HSV-1 KOS (Fig. 5B). It is notable that Vero cells, a monkey kidney cell line often used to prepare viral stocks of HSV-1, produce a viral titer almost a log higher than undifferentiated SH-SY5Y cells (Fig. 5B). We also compared HSV-1 strains McKrae and KOS in a high-MOI infection of neuronal cells and Vero cells and found no difference in the replication rates over this single round of replication (data not shown). These data



**FIG 4** Neuronal SH-SY5Y cells release less extracellular virus during lytic HSV-1 infection. (A) Single-step growth curves of viral replication kinetics in differentiated SH-SY5Y neuronal cells (blue), undifferentiated SH-SY5Y cells (red), and SK-N-SH cells (gray). Each cell type was infected with HSV-1 McKrae at an MOI of 10. Titers are plotted as log PFU per 100,000 cells. Cell-free viral titers, cell-associated viral titers, and combined total viral titers include three biological replicates. A 2-way analysis of variance (ANOVA) was performed for each graph: for cell-free virus, \*,  $P = 0.0229$ ; \*\*\*,  $P = 0.004$ ; for cell-associated virus, \*,  $P = 0.0452$ . Each graph displays the average from the three replicates, and the error bars represent the standard errors of the means (SEM). (B) Time-lapse images reveal morphological changes in differentiated SH-SY5Y neuronal cells, undifferentiated SH-SY5Y cells, and SK-N-SH cells during infection with HSV-1 McKrae at a high MOI (10). Initial changes in neuronal cell morphology begin at 6 hpi, with clear neurite retraction by 12 hpi. At 24 hpi, neuronal morphology is markedly different and few neurites remain. Following viral infection in the undifferentiated SH-SY5Y cells and SK-N-SH cells, morphological changes are not visible until 12 hpi. By 24 hpi, the undifferentiated SH-SY5Y cells appear to all be infected and rounded up. The SK-N-SH cells appear to be more robust, with only about 50% of cells rounded up by 24 hpi. The scale bar represents 25  $\mu$ m. Images are representative of those from three biological replicates. (C) Procaspase 3 expression data demonstrate cleavage and activation of caspase 3 in differentiated SH-SY5Y neuronal cells, undifferentiated SH-SY5Y cells, and SK-N-SH cells over a time course of 24 hpi with HSV-1 McKrae (MOI of 10). Sorbitol-induced osmotic shock serves as a positive control for activation of caspase 3. Blots are representative of those from two biological replicates.

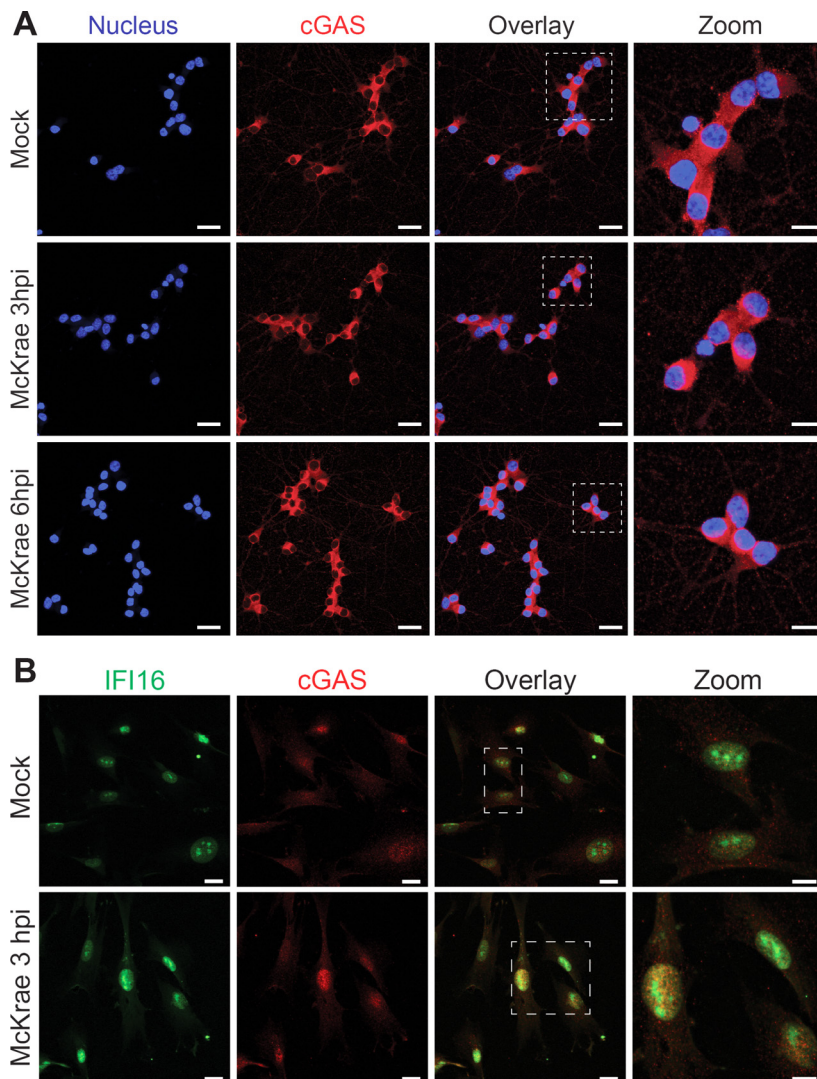




**FIG 5** Neuronal SH-SY5Y cells reveal distinct phenotypes of cell-to-cell spread for different strains of HSV-1. Following a low-MOI infection with sample collection at 24, 48, and 72 hpi, neuronal SH-SY5Y cells (A) but not undifferentiated SH-SY5Y cells or Vero cells (B) distinguish between two phenotypically distinct viral strains. For these multistep growth curves, all three cell types were infected with HSV-1 McKrae (green) or HSV-1 KOS (orange) at an MOI of 0.01 in the presence of 0.1% pooled human serum. Data include three biological replicates, and error bars represent the SEM. Multiple *t* tests were performed for neuronal SH-SY5Y cells, with alpha of 5.0%. \*, *P* = 0.04.

suggested that cell-to-cell-spread assays, such as this low-MOI (multistep) growth curve in differentiated SH-SY5Y neuronal cells, may prove useful in screening HSV-1 strains for potential differences in their abilities to replicate and spread in a neuron-specific manner.

**Differentiated neuronal cells have a unique pattern of cGAS expression compared to MRC5 cells before and during HSV-1 infection.** Prior research has shown that IFI16 and cGAS can act together to detect HSV-1 viral DNA in the nuclei of epithelial cells following HSV-1 infection (19, 20). However, the expression of these factors in human neurons has not yet been investigated. Through confocal microscopy, we found that differentiated SH-SY5Y neuronal cells expressed cGAS both before and during HSV-1 infection (Fig. 6A). Expression of IFI16 was not sufficiently above the level of neuronal autofluorescence for a robust comparison, and was therefore excluded from further analysis (data not shown). cGAS localized to the cytoplasm of uninfected differentiated SH-SY5Y neuronal cells, which was consistent with prior observations in epithelial cells. No significant change in cGAS expression levels (Fig. 3A) or localization (Fig. 6A) occurred following infection with HSV-1 McKrae at 3 or 6 hpi. These neuronal localization data were different from what has been reported for cGAS expression in epithelial cell types. To confirm the efficacy of the cGAS and IFI16 detection used in this study, we examined both proteins in human lung fibroblasts (MRC5) (Fig. 6B). MRC5 fibroblasts expressed IFI16 in the nucleus and cGAS in the cytoplasm prior to HSV-1 infection. Following viral infection, we observed both IFI16 and cGAS in the nuclei of these epithelial cells, as has been shown in other epithelial cell types. Taken together, these data suggested that terminally differentiated SH-SY5Y cells provide a homogeneous and reproducible model system for the investigation of HSV-1 infection and cGAS antiviral signaling from a neuronal perspective *in vitro*.



**FIG 6** Differentiated neuronal cells have a unique pattern of cGAS expression compared to MRC5 cells before and during HSV-1 infection. Differentiated neuronal cells express the antiviral protein cGAS in the cytoplasm and in neurites both before and after HSV-1 infection, while MRC5 cells express cGAS and IFI16 in the nucleus following HSV-1 infection. (A) Terminally differentiated SH-SY5Y neuronal cells were infected at an MOI of 10 with HSV-1 McKrae and then fixed and processed for cGAS expression at 3 hpi or 6 hpi. Scale bars represent 20  $\mu\text{m}$ . The zoomed-in images were acquired with a 168 $\times$  objective, and scale bars represent 10  $\mu\text{m}$ . (B) MRC5 fetal lung fibroblasts were infected at an MOI of 10 with HSV-1 McKrae and then fixed and processed for immunofluorescence at 3 hpi. Scale bars represent 20  $\mu\text{m}$ . The zoomed-in images were acquired with a 156 $\times$  objective, and scale bars represent 10  $\mu\text{m}$ . Images were taken with an Olympus FV10i confocal microscope with a 60 $\times$  objective.

## DISCUSSION

Here we describe an *in vitro* model of differentiated human neuronal cells that serves as a facile system for biochemical and molecular dissection of HSV-1-neuron interactions. We characterized the neuronal phenotype of terminally differentiated SH-SY5Y cells and compared these data to those for undifferentiated SH-SY5Y progenitor cells and the parental SK-N-SH cell line. We showed that terminally differentiated SH-SY5Y cells possessed neuronal morphology that was distinct from that of undifferentiated SH-SY5Y cells or SK-N-SH cells. Specifically, we demonstrated that terminally differentiated SH-SY5Y cells expressed proteins associated with a mature neuronal phenotype, including the appropriate localization of markers for axons, dendrites, and synapses. This model yields homogeneous populations of human neuronal cells, which

provide a species-matched platform with which to explore the molecular and cellular mechanisms of viral infection in humans.

Consistent with published data (45, 46), we found that terminally differentiated SH-SY5Y cells expressed neuronal proteins and appeared to have both axons (via p-NF-H) and dendrites (via MAP-2). MAP-2 associates with tubulin to help promote the assembly of microtubules in both axons and dendrites of neurons (45). *In vivo*, the axonal localization of p-NF-H occurs only after the neurofilaments have entered into mature axons (60); likewise, p-NF-H is only observed in the terminally differentiated SH-SY5Y neuronal cells and not in undifferentiated SH-SY5Y precursor cells. Differentiated SH-SY5Y neuronal cells appeared to have synapses, based on the expression and localization of SV2 and the high total protein levels of synaptophysin (SY38) (Fig. 2 and 3) (61, 63).

Herpesvirus infection triggers activation of stress response genes and pathogen recognition receptors (19, 20, 71). Protein kinase B, also known as AKT, is a serine/threonine kinase that is part of the phosphatidylinositol 3-kinase (PI3-K) pathway (72, 73). Both AKT and PI3-K signaling are required for RA-induced differentiation of neuroblastoma cells (74). Neuronal SH-SY5Y cells had significantly higher expression levels of p-AKT than undifferentiated SH-SY5Y cells (Fig. 3). Since AKT is known to undergo autophosphorylation and activation (p-AKT) in response to stimulation of Trk from neurotrophin binding (52, 72, 73), the increased level of p-AKT in neuronal SH-SY5Y cells (Fig. 3) likely reflects neuronal differentiation and Trk activation (75). Increased p-AKT levels following viral infection (Fig. 3) match prior findings by other groups (76–78). Activated p-AKT functions in apoptosis, protein synthesis, and cell growth (79–81), and viruses, including HSV-1, have been shown to hijack this pathway during infection (76–78, 82, 83). Prior data have shown that continuous signaling via the PI3-K pathway and p-AKT in superior cervical ganglion-derived neurons is also critical for the maintenance of HSV-1 latency *in vitro* (65, 84, 85). This provides an exciting avenue for further study, as a neuronal system primed with high levels of p-AKT may facilitate development of an improved model for HSV-1 latency.

We also found that neuronal SH-SY5Y cells expressed the antiviral sensor cGAS (Fig. 3A and Fig. 6A). This protein elicits activation of interferon regulatory factor 3 (IRF-3) via the stimulator of interferon gene (STING)-dependent pathway, resulting in activation of the type I interferon (IFN) pathway and production of IFN- $\beta$  (16, 86). cGAS has been shown to detect many viral pathogens in the cytosol (66) and is therefore an important component of the host antiviral defense system. Additionally, cGAS is known to interact with activated p-AKT, which phosphorylates cGAS and inhibits activation of the downstream interferon pathway (87). It is hypothesized that in epithelial cells, IFI16 detects and binds directly to HSV-1 DNA in the nucleus (18), with subsequent interaction of cGAS to stabilize IFI16's interaction with the viral DNA (20). We showed for the first time that the antiviral factor cGAS is expressed in human neuronal cells (Fig. 3 and 6A). We also saw that cGAS expression and localization in neuronal cells was different from what has been reported for epithelial cells, such as MRC5 cells (Fig. 6B) (15, 19, 20). cGAS does not appear to translocate to the nucleus of neuronal cells following HSV-1 infection (Fig. 6A). It is possible that the neuroblastoma origin of the SH-SY5Y cells influences their expression and/or localization of cGAS. More work is needed to determine how cGAS is expressed in different types of neurons and how it contributes to the intrinsic and innate responses of the nervous system to infection.

At present, our understanding of viral neuroinvasion is based on mouse models of HSV-1 infection. The herpesvirus field lacks an *in vitro* proxy measurement for neuroinvasion *in vivo*. *In vivo* comparisons are costly in terms of time, labor, and animal use, and thus, these animal models are not universally employed for every comparison of HSV strains. An *in vitro* model that could serve as a preliminary assessment of neuronal spread and potential for neuroinvasion would enable further cross-comparison of viral strains. We found that using differentiated SH-SY5Y neuronal cells and a low-MOI infection, we are able to distinguish between HSV-1 McKrae and HSV-1 KOS. The HSV-1 McKrae strain reached a 10-fold-higher titer in differentiated SH-SY5Y neuronal cells

than the HSV-1 KOS strain (Fig. 5A). Multiple groups have demonstrated that HSV-1 McKrae is a highly neuroinvasive virus, capable of penetrating into the murine central nervous system and inducing subsequent mortality at a high rate (67, 68, 88). In contrast, HSV-1 KOS was found to be less neuroinvasive, and when introduced at the periphery, it did not induce significant mortality (8, 68, 89). This suggests that the reduced cell-to-cell spread of HSV-1 KOS in neuronal cells may contribute to the observed differences in viral strain neuroinvasion. In addition, HSV-1 KOS is known to carry a defective copy of the viral Us9 protein, which contributes to virion transport in axons (90–92). We do not yet know whether these *in vitro* and *in vivo* strain differences are linked, and therefore, this is an exciting area for future investigation. Moreover, the identical multistep growth curves of HSV-1 McKrae and KOS in undifferentiated SH-SY5Y cells and in Vero cells (Fig. 5B) further emphasize that epithelial cell types are not sufficient for detecting differences in neuronal spread *in vitro*.

Within the herpesvirus field, a diverse array of models have been used to study the latent stage of HSV-1 infection (32, 34, 67, 68, 93). While these models have established the foundation of our understanding of latency and reactivation, there are caveats associated with these systems. For example, nonhuman animal models are frequently used to study a species-specific virus that has coevolved with its human host (33, 67, 68, 93). Existing *in vitro* models for the study of HSV-1 latency have yielded promising insights into its molecular mechanisms, but with caveats such as rodent species origins (34, 65, 93–97) and variable differentiation of human neuronal progenitors (31, 98). Terminally differentiated SH-SY5Y cells provide a homogeneous, mature population of human neuronal cells, which may be ideal for adaptation to the *in vitro* latency models currently applied to rodent neurons (34, 65, 94–97). This idea is further supported by the constitutively high p-AKT levels observed in differentiated SH-SY5Y neuronal cells, since p-AKT is known to support HSV-1 latency in other cell types (65, 84, 85).

In conclusion, this model provides a simplified view in which neuronal cells are the focus, which allows for detailed analyses of virus-neuron interactions *in vitro*. Using this system, we have detected that neuronal cells and epithelial progenitor cells mount different responses to HSV-1 infection. These differences include a more limited release of extracellular virus by differentiated SH-SY5Y neuronal cells, as well as their ability to reveal differences between HSV-1 strains in neuronal cell-to-cell spread phenotypes. These findings warrant future studies to explore how these distinct cellular responses relate to the human biology of HSV-1 infection.

## MATERIALS AND METHODS

**Cells and viruses.** Vero African green monkey kidney cells (ATCC; CCL-81), SK-N-SH neuroblastoma cells (ATCC; HB11), and the SK-N-SH subcloned SH-SY5Y cell line (ATCC; CLR-2266) were maintained at 37°C with 5% CO<sub>2</sub>. Vero cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS), supplemented with penicillin-streptomycin (Pen/Strep; Life Technologies-Gibco) and L-glutamine (Thermo Fischer Scientific-HyClone). SK-N-SH cells were cultured in Eagle's minimal essential medium (EMEM) using 10% heat-inactivated fetal bovine serum (HIFBS), supplemented with Pen/Strep and L-glutamine. Undifferentiated SH-SY5Y and differentiated SH-SY5Y cells were cultured as previously described (53) in a protocol based on those of Christensen et al. (46) and Encinas et al. (45). Briefly, undifferentiated SH-SY5Y cells were plated in EMEM formulated with HIFBS, Pen/Strep, L-glutamine, and 10 μM RA for 7 days, with medium changes every other day. Cells were then transitioned through two additional medium formulations to slowly deplete serum and add in neurotrophic factors, including brain-derived neurotrophic factor (BDNF) (53). The final medium formulation was neurobasal medium (Life Technologies-Gibco) with Glutamax (Life Technologies-Gibco), Pen/Strep, B-27 (Thermo Fischer Scientific), 1 M KCl, dibutyryl cyclic AMP (Sigma), and BDNF (Sigma). The cells were lightly trypsinized and replated onto new 35-mm<sup>2</sup> dishes on days 7 and 10 of the protocol to preferentially select for transfer and survival of the neuron-like cells. Cells were terminally differentiated at 2.5 weeks and ready to use.

HSV-1 strain McKrae was provided by Lynda Morrison (69, 99). KOS<sub>63D</sub> virus was obtained from Richard Dix (8, 100) and is referred to as KOS throughout. HSV-1 strain F-GS2822, a bacterial artificial chromosome (BAC)-derived virus, was provided by Sarah Antinone and Greg Smith (101). This virus is referred to as F<sub>RED</sub> throughout. Viral stocks were grown and titers were determined on Vero cells using DMEM with 2% FBS and antibiotics.

Unless stated otherwise, infections using HSV-1 McKrae, HSV-1 KOS, and HSV-1 F<sub>RED</sub> were performed as follows. Stocks of virus were freeze-thawed three times and sonicated in a cup holder at 80% amplitude for 10 s on/off using a Q500 ultrasonic processor (QSonica). Virus dilutions were made in

cell-appropriate media (listed above) based on average cell counts and the desired MOI. Cells were infected with the selected virus strain and MOI or mock infected using medium specific for each cell type in a total volume of 200  $\mu$ l per 35-mm<sup>2</sup> plate. Following HSV infection or mock infection, plates were incubated at 37°C for 1 h, with rocking every 15 min. Inocula were aspirated and replaced with fresh cell-appropriate media.

**Antibodies.** The antibodies, sources, hosts, assays, dilutions, and protein targets were as follows: glyceraldehyde-3 phosphate dehydrogenase (GAPDH), Abcam, rabbit polyclonal, WB, 1:2,500, and glyceraldehyde-3 phosphate dehydrogenase; HSV-1 gE, Harvey Friedman, mouse, WB, 1:1,000, and HSV-1 glycoprotein E; AKT, Cell Signaling Technology, rabbit monoclonal, WB, 1:1,000, and protein kinase B; p-AKT, Cell Signaling Technology, rabbit monoclonal, WB, 1:2,000, and phosphorylated protein kinase B; MAP-2, Cell Signaling Technology, rabbit polyclonal, IF, 1:500, and microtubule-associated protein 2; SY38, Abcam, mouse, WB, 1:500, and synaptophysin; GAP-43, Santa Cruz Biotechnology, mouse, WB, 1:1,000, and growth-associated protein 43; IFI-16, David Knipe, mouse monoclonal, IF, 1:50, and IFN- $\gamma$ -inducible protein 16; cGAS, David Knipe, rabbit polyclonal, WB/IF, 1:1,000/1:50, and cyclic GMP-AMP synthase; caspase 3, Cell Signaling Technology, rabbit polyclonal, WB, 1:1,000, inactive, and full-length procaspase 3; SMI-31, Covance, mouse monoclonal, IF, 1:500, and phosphorylated NF-H; SMI-32, Covance, mouse, IF, 1:500, and unphosphorylated NF-H; SV2, Developmental Studies Hybridoma Bank, mouse, IF, 1:250, and synaptic vesicle protein 2; anti-mouse [F(ab')<sub>2</sub>] Alexa Fluor 488, Jackson ImmunoResearch, donkey, IF, 1:200, and secondary antibody—IF; anti-rabbit [F(ab')<sub>2</sub>] Alexa Fluor 647, Jackson ImmunoResearch, donkey, IF, 1:200, and secondary antibody—IF; anti-mouse [F(ab')<sub>2</sub>] Alexa Fluor 647, Jackson ImmunoResearch, donkey, IF, 1:200, and secondary antibody—IF; anti-rabbit [F(ab')<sub>2</sub>] Alexa 488, Jackson ImmunoResearch, donkey, IF, 1:200, and secondary antibody—IF; goat anti-rabbit, Jackson ImmunoResearch, goat, WB, 1:20,000, and secondary antibody—WB; goat anti-mouse, Jackson ImmunoResearch, goat, WB, 1:20,000, and secondary antibody—WB.

**Imaging and immunofluorescence.** Phase-contrast images of uninfected and infected cells were obtained on an inverted epifluorescence microscope (Nikon Eclipse Ti). Images were acquired at a magnification of  $\times 20$  in the bright-field channel. Time-lapse images were taken using an inverted epifluorescence microscope (Nikon Eclipse Ti) with a stage-top incubator (Tokai Hit).

To assess expression and localization of neuronal markers (Fig. 2), undifferentiated SH-SY5Y cells and terminally differentiated SH-SY5Y neuronal cells (53) were grown on ECM-coated glass coverslips. Cells were fixed in 4% paraformaldehyde, rinsed with 1 $\times$  phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100–PBS, and blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) in 0.1% Triton X-100–PBS. Cells were then incubated with primary antibodies (see “Antibodies” above) diluted in 10% donkey serum–0.1% Triton X-100–PBS overnight at 4°C. Coverslips were washed with 0.1% Triton X-100–PBS and then incubated with fluorescence-conjugated secondary antibodies (see “Antibodies” above) diluted in 10% donkey serum–0.1% Triton X-100–PBS for 2 h at room temperature. Cells were washed with 0.1% Triton X-100–PBS, rinsed three times with PBS, and then mounted using ProLong Gold plus 4',6-diamidino-2-phenylindole (DAPI) antifade mounting reagent (Invitrogen).

Assessments of expression and localization of the immune mediators IFI16 and cGAS (Fig. 6) were performed as previously described (20). Briefly, MRC5 fibroblasts and terminally differentiated SH-SY5Y neuronal cells (53) were infected at an MOI of 10 with HSV-1 McKrae in a total volume of 400  $\mu$ l and fixed in 2% paraformaldehyde at 3 hpi or 6 hpi (neuronal cells only). All wash steps and incubations in primary and secondary antibodies were performed as described above, with the use of Hoechst nuclear stain (1:10,000) diluted in 10% donkey serum–0.1% Triton X-100–PBS. Coverslips were then mounted on slides with ProLong Gold antifade reagent (Invitrogen).

All fluorescence images were acquired using an Olympus FV10i confocal microscope with a 60 $\times$  objective and digital zoom as indicated in the text and figure legends. Each image is presented as a maximum projection with background subtraction performed in ImageJ (rolling ball radii = 50 for neuronal cells and 80 for MRC5 cells). Brightness and contrast were adjusted equally for each figure in Adobe Photoshop. To compare expression and localization of neuronal markers, identical laser settings were applied for each SH-SY5Y image pairing (Fig. 2).

**Western blotting.** Soluble protein was separated from total cell lysate as previously described (102). Briefly, cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with Pierce protease and phosphatase inhibitor minitables (Thermo Scientific). Cells were scraped and combined and then sonicated in a cup holder at 80% amplitude for 4 s on/off using a Q500 ultrasonic processor. Lysates were rocked for 15 min at 4°C and centrifuged at 12,500  $\times g$  for 10 min at 4°C. Supernatant was collected and total protein was quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific) on a NanoDrop 2000c spectrophotometer.

Twenty micrograms of protein from each sample was resolved in 7.5 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) minigels (Miniprotean; Bio-Rad) and transferred to a 0.45- $\mu$ m or 0.20- $\mu$ m nitrocellulose membrane (Amersham GE Healthcare) using a Trans-Blot SD semidry electrophoresis transfer cell (Bio-Rad) at 15 V for 17 to 25 min. Membranes were blocked in either 5% nonfat dry milk or 3% bovine serum albumin (BSA) prepared in Tris-buffered saline (1 M, pH 7.4), 154 mM NaCl, and 0.2% Tween 20 (TBS-T buffer) for 1 h at room temperature. Primary antibodies were diluted in either 1% milk plus TBS-T or 3% BSA plus TBS-T and incubated overnight at 4°C (see “Antibodies” above). Membranes were washed three times with TBS-T buffer for 20 min each time and then incubated in a 1:20,000 dilution of species-appropriate secondary antibody in either 1% milk plus TBS-T or 3% BSA plus TBS-T for 1 h at room temperature (see “Antibodies” above). Membranes were washed as described above and visualized using enhanced chemiluminescence substrate, SuperSignal West Pico substrate, or SuperSignal West Dura substrate (Thermo Scientific).

For infected-cell Western blots, the above-described protocol was repeated following initial infection with an MOI of 10 using HSV-1 McKrae. Following the infection procedure detailed above, the inoculum was aspirated and replaced with cell-appropriate medium. Pairs of uninfected and infected cell lysates were harvested at 6 hpi.

To assess activation of the caspase cascade and subsequent apoptosis in neuronal SH-SY5Y cells and the epithelial progenitor cells, we examined cleavage of procaspase 3 protein by Western blotting (Fig. 4B). As a positive control for procaspase 3 cleavage, we induced osmotic shock by treating cells with 1.5 M sorbitol for 1 h, followed by 2 h of incubation at 37°C, as previously described (103).

Protein expression levels were quantified using ImageQuant TL8.1 software (GE Healthcare Life Sciences). Regions of interest were drawn around each sample lane, and then rolling-ball subtraction was used to eliminate background signal. The size of the rolling ball used for background subtraction on all Western blot replicates was between 55 and 70 on a parameter scale of 0 to 10,000. Total protein expression present in each sample lane was determined, and each biological replicate for all protein targets of interest was normalized to the matched GAPDH loading control lane obtained on the same day. p-AKT levels were normalized to the matched AKT expression levels for each biological replicate. Ratios of each protein of interest to GAPDH expression were plotted for both undifferentiated and differentiated SH-SY5Y cells (Fig. 3B). For infected Western blot quantitation, ratios of each protein of interest to GAPDH were normalized to the uninfected expression levels for each protein of interest (Fig. 3C).

**Single-step and multistep growth curves.** Neuronal cultures were obtained by initial plating of undifferentiated SH-SY5Y cells at a density of 200,000 or 400,000 cells per 35-mm<sup>2</sup> plate. These were differentiated as previously described (45, 46, 53). The average number of neuronal cells obtained following the 2.5-week differentiation procedure was typically between 30,000 and 100,000 per 35-mm<sup>2</sup> plate. For the single-step growth curve comparison of all three cell types (Fig. 4A), undifferentiated SH-SY5Y cells and SK-N-SH cells were plated 24 h prior to infection at a density of 50,000 or 100,000 per 35-mm<sup>2</sup> plate. On the day of infection, three plates from each cell type were used for cell counting and subsequent virus dilution calculations. Cells were infected at an MOI of 10 and after a 1-h incubation with rocking, the inoculum was replaced and infections were allowed to proceed for 24 h. Both cell-free virus and cell-associated virus were collected during the single round of replication. To harvest the cell-free fraction, medium from infected cells was collected and centrifuged at 1,000 × *g* for 2 min and the supernatant was saved. To collect the cell-associated fraction, infected-cell monolayers were scraped and harvested, resuspended in media, and combined with pelleted cells from the supernatant collection. Both the cell-free and cell-associated samples were freeze-thawed three times prior to determination of virus titers on Vero cells as described above.

For the multistep growth curves (Fig. 5), differentiated SH-SY5Y cells, undifferentiated SH-SY5Y cells, and Vero cells were infected with an MOI of 0.01, with prior counting of three plates to determine the average cell density per plate. Following infection and a 1-h incubation with rocking, the inoculum was replaced with fresh medium containing 0.1% pooled human serum from male AB plasma (Sigma; H4522) and infections were allowed to proceed for 72 h (104, 105). During this time, both cell-free virus and cell-associated virus were collected at 24, 48, and 72 hpi. To harvest the cell-free fraction, medium from infected cells was collected and centrifuged at 5,000 × *g* for 5 min and the supernatant was saved. To collect the cell-associated fraction, infected cell monolayers were scraped and harvested, resuspended in medium, and combined with pelleted cells from the supernatant collection. The samples were thawed, and titers on Vero cells were determined as described above.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00958-17>.

**SUPPLEMENTAL FILE 1**, MOV file, 9.4 MB.

**SUPPLEMENTAL FILE 2**, MOV file, 5.8 MB.

**SUPPLEMENTAL FILE 3**, PDF file, 0.1 MB.

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