

Optimisation of lytic herpes simplex virus infection in human induced pluripotent stem cell derived cortical neurones

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Abstract

Herpes simplex virus (HSV)-1 infection of cortical neurones is a leading cause of encephalitis. While we have substantial knowledge about the molecular virology of HSV-1 lytic infection in cells of the periphery, like keratinocytes or fibroblasts, we know much less about infection of human neurones owing to the challenges of working with neuronal cell-based models. Here we demonstrate the use of a human induced pluripotent stem cell (iPSC)-derived cortical neurone model (i3Neurones) for HSV-1 infection. i3Neurones are highly scalable and can be rapidly and efficiently differentiated into an isogenic population of cortical glutamatergic neurones. We show that i3Neurones support the full HSV-1 lytic replication cycle. We present an optimised protocol for the infection of i3Neurones with HSV-1 that allows their synchronous infection at near-100% efficiency, and optimised fixation methods that preserves organelle and neurite structure for immunocytochemistry analysis. Our study highlights i3Neurones as a robust, scalable platform for microscopy and biochemical studies of HSV-1 and other neurotropic pathogens.

Data summary

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

29 Introduction

30 Neuronal virus infections cause severe pathology. HSV-1 is the leading cause of viral
31 encephalitis [1], causing 70% mortality in untreated patients and up to 19% in patients treated
32 with antivirals, with survivors often suffering severe neurological sequelae [2]. Similarly,
33 infection with enteroviruses such as EV-A71 cause encephalitis and acute flaccid paralysis [3];
34 Zika virus and Oropouche virus infection in adults can cause Guillain-Barré syndrome [4, 5]; and
35 congenital Zika virus infection can cause microcephaly, decreased brain tissue, plus ocular and
36 osteoskeletal abnormalities [6]. Furthermore, it is increasingly clear that infection with
37 neurotropic viruses like HSV is a risk factor for developing common neurodegenerative diseases
38 [7–9]. It is therefore important to identify robust and appropriate human cell-based systems to
39 study the molecular basis of neuronal infection by HSV-1 and other neurotropic pathogens.

40 Multiple different systems have been used for the study of neuronal HSV-1 infection, especially
41 in the context of latency (reviewed in [10]). *Ex vivo* infection of rat or mouse-derived ganglia that
42 represent the natural site of HSV-1 latency are widely used [11–13], although the number of
43 neurones that can be isolated even from a large number of animals is limited [14] and there are
44 differences in interactions between HSV-1 and mouse versus human immune responses [15,
45 16]. *Ex vivo* studies of human neurones is possible using post-mortem specimens [17, 18], but
46 availability and the capacity for genetic manipulation of the specimens is limited. Human
47 embryonic or induced pluripotent stem cells (iPSCs) allow interrogation of infection following
48 differentiation into neural stem cells, neurones or other glial cell types [19–23]. However,
49 differentiation timescales can be long and the procedures labour-intensive. Human stem-cell
50 derived organoids represent a powerful model for studying the functional consequences of
51 HSV-1 lytic and latent infection in human neural stem cells, neurones and glia [24–28]. While
52 excellent for transcriptomic analysis [25, 28], these models are not well suited to high resolution
53 proteomics studies of infection such as quantitative temporal viromics [29], which require large
54 numbers of homogenous cells ($\geq 1 \times 10^7$) and high levels of synchronous infection ($\geq 90\%$) [30,
55 31].

56 Scalable cancer-derived neuroblastoma cell lines like SH-SY5Y are widely used for HSV-1
57 infection studies [21, 32, 33] but they have complex chromosomal aberrations [34, 35], are
58 highly sensitive to the differentiation procedure used and yield mixed morphology populations
59 [36]. Lund human mesencephalic (LUHMES) cells have been developed as models to study
60 HSV-1 latency [37] and host shutoff during lytic infection [38]. Differentiated LUHMES resemble
61 post-mitotic dopaminergic neurones [39] and they represent a powerful homogenous cell-
62 based system for studies of neuronal infection. However, LUHMES cells are derived from the
63 midbrain mesencephalon [40] whereas herpes simplex encephalitis (HSE) is generally localised
64 to the temporal lobes [41]. As we know that innate immune programs differ between different
65 classes of neurone [42], there is a need for additional scalable systems for the study of HSV-1
66 infection in the cerebral cortex.

67 Differentiation of human iPSCs via the expression of integrated transcription factors represents
68 a promising approach to rapidly obtain isogenic populations of differentiated neuronal and
69 other cell types [43]. For example, human iPSCs expressing the neuronal transcription factor
70 Neurogenin3 (NGN3) [43] can be differentiated into sensory human neurones that have been
71 used to characterise miRNAs and neuronal factors that regulate the efficiency of HSV-1 lytic
72 replication or establishment of latency [44, 45]. Recently, a comprehensive analysis has shown
73 that sensory neurones differentiated via NGN3 expression support synaptic firing plus lytic
74 replication, latency and reactivation of HSV-1 [46]. However, given the clinical importance of

HSE it is also necessary to have scalable, tractable systems to probe lytic infection of cortical neurones. This can be achieved via differentiation driven by Neurogenin2 [47]. Human iPSCs with doxycycline-inducible *Ngn2* in a safe-harbour locus [48] can be differentiated in 14 days into cortical glutamatergic neurones with close to 100% efficiency using a simple two-step protocol [49]. These integrated, inducible, and isogenic iPSCs (i3Neurones) express markers typical of cortical glutamatergic neurones as demonstrated by multiple techniques. Immunofluorescence analysis shows the presence of the presynaptic vesicular glutamate transporter VGLUT1 and the postsynaptic AMPA-type glutamate receptor formed by GluR2/3 subunits, along with general pan-neuronal markers including β III tubulin, MAP2 and NeuN [48]. Protein and mRNA profiling demonstrating widespread expression of synaptic AMPA-type receptors, glutamate transporters, and key developmental proteins like the CUX1 transcription factor that is widely expressed in pyramidal neurones across layers of the cerebral cortex [48, 50–52]. Furthermore, mature i3Neurones exhibit robust synchronous neuronal firing [50] and their amenability to genetic manipulation [50, 53] makes them suitable for precisely targeted functional studies. The ability to generate a homogenous isogenic population via activation of a stably-integrated master transcriptional regulator reduces experimental variability that can confound the interpretation of genome- or proteome-wide screening experiments [48], making these i3Neurones a robust platform for molecular discovery research.

Here we present optimisation and initial characterisation of lytic HSV-1 infection of i3Neurones, expanding the toolkit for biochemical and functional characterisation of neuronal HSV-1 infection.

Methods

Stem cell culture

Human fibroblast-derived iPSCs containing a doxycycline-inducible *Ngn2* transcription factor and an inactivated (dead) Cas9 gene in a safe-harbour locus [54] were provided by Michael Ward (National Institutes of Health, USA) and cultured as per [49]. Briefly, iPSCs were maintained in a 5% CO₂ humidified atmosphere at 37°C in dishes pre-coated with hESC-qualified Matrigel (Corning 354277) diluted 1:100 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco 11330032). Essential 8 medium (Gibco A1517001) was used for 24 hr culture and Essential 8 Flex medium (Gibco A2858501) for 72 hr culture, and colonies were subcultured by dissociation using 0.5 mM EDTA in PBS. iPSCs were dissociated to single-cell suspension with StemPro Accutase Cell Dissociation Reagent (Gibco A1110501) and seeded in medium supplemented with 50 nM Chroman1 (Rho-associated protein kinase (ROCK) inhibitor; Bio-Techne 7163/10).

Neuronal differentiation

Differentiation of iPSCs into i3Neurones followed a two-step protocol of differentiation and maturation as outlined in [49]. In summary, $1.5\text{--}1.8 \times 10^7$ iPSCs were seeded following Accutase dissociation in a Matrigel-coated 15 cm dish (Day 0) and incubated for three days in Induction Medium (IM): DMEM/F-12, supplemented with $1 \times$ N2 supplement (Gibco 17502048), $1 \times$ non-essential amino acids (NEAA, Gibco 11140050), $1 \times$ L-glutamine (Gibco 25030081), plus 2 $\mu\text{g}/\text{mL}$ doxycycline (Sigma Aldrich D3072) to induce *Ngn2* expression. The medium was changed daily, being supplemented with 50 nM Chroman 1 for the first day of differentiation. At day 3 the i3Neurone precursor cells were dissociated with Accutase and frozen at -80°C in

cryopreservation media comprising 90% (v/v) KnockOut Serum Replacement (Gibco 10828010) and 10% (v/v) DMSO before being stored in liquid nitrogen.

Day 3 i3Neurone precursor cells were cultured for a further 11 days (to day 14) in cortical neurone (CN) culture medium comprising Neurobasal Plus Medium (Gibco A3582901) supplemented with 1 × B27 supplement (Gibco A3582901), 10 ng/mL Brain Derived Neurotrophic Factor (BDNF, PeproTech 450-02), 10 ng/mL Neurotrophin-3 (NT-3, PeproTech 450-03) and 1 µg/mL Laminin (Gibco 23017015). Frozen cells were thawed rapidly, diluted 10-fold in DMEM/F12 medium, pelleted by centrifugation (300 × g, 5 min), resuspended in CN medium supplemented with 2 µg/mL doxycycline and counted using a Countess II FL automated cell counter (Invitrogen). i3Neurons were seeded on plates coated with 100 µg/mL poly-L-ornithine (PLO) in half of the final culture volume of CN medium, incubated for 15 min at room temperature to allow cells to settle evenly, and then the remaining half of the medium was added before returning the cells to the incubator. When first seeding the cells the CN medium was supplemented with 2 µg/mL doxycycline, and half the volume of CN medium was replaced every three days until day 14, at which time the i3Neurons were used for experiments. At all stages, cells were cultured in a 5% CO₂ humidified atmosphere at 37°C, and final differentiated neurones were confirmed as being free of mycoplasma.

Non-neuronal cell culture

Vero (ATCC CRL-1586), U2OS (ATCC HTB-96) and U2OS pUL21-BirA*-HA cells (see below) were grown in DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and 2 mM L-glutamine (complete DMEM) in a 5% CO₂ humidified atmosphere at 37°C. All cells were frequently tested for mycoplasma and confirmed as mycoplasma-free.

U2OS cells expressing pUL21 tagged with an abortive biotin ligase (BirA*) [55] were generated by co-transfection of Flp-In T-REx U2OS cells provided by Gopal Sapkota (University of Dundee, UK) [56] with pOG44 (Invitrogen) and pcDNA5/FRT/TO (Invitrogen) encoding codon-optimised HSV-1 pUL21 (UniProt F8RG07) [57] with a C-terminal BirA* plus HA epitope tag. At 72 h post-transfection the culture medium was replaced with fresh medium containing 200 µg/mL hygromycin B and 3 µg/mL blasticidin. Selection of hygromycin and blasticidin resistant cells was allowed to proceed for 19 days, with medium being refreshed every 2-3 days as required. pUL21-BirA*-HA expression was induced by addition of 2 µg/mL doxycycline 24 h prior to use.

Antibodies

Antibodies were used for immunofluorescence microscopy (IF), immunoblotting and virus neutralisation. See Table 1 for full details of antibodies and dilutions used.

Table 1. Antibodies used in this study.

Antibody	Source	Dilution for immunocytochemistry	Dilution for immunoblotting
Mouse monoclonal anti-gD (LP2)	[58]	1:9	–
Mouse monoclonal anti-gD (LP14)	[58]	–	1:20
Mouse monoclonal anti-ICP4	ATCC 58S	1:9	–
Rabbit monoclonal anti-GOPC	Abcam Ab133472	–	1:1000
Mouse monoclonal anti-VP5 (DM165)	[59]	–	1:9
Mouse monoclonal anti-GAPDH	GeneTex GTX28245	–	1:5000
Rabbit polyclonal anti-OCT4	NEB 2750s	–	1:1000

Rabbit polyclonal anti- β III Tubulin	Abcam Ab18207	–	1:5000
Chicken polyclonal anti- β III Tubulin	NB100-1612	1:1000	–
Mouse monoclonal anti-TAU	Abcam Ab80579	1:50000	1:50000
Rabbit polyclonal anti-MAP2	Merck Ab5622	1:1000	–
Sheep polyclonal anti-TGN46	Bio-Rad AHP500G	1:200	–
Mouse monoclonal anti-pUL21 (1F10)	[60]	–	1:1
Goat anti-mouse 800	LI-COR 926-32210	–	1:10000
Goat anti-mouse 680	LI-COR 926-68020	–	1:10000
Goat anti-rabbit 800	LI-COR 926-32213	–	1:10000
Goat anti-rabbit 680	LI-COR 926-68023	–	1:10000
Goat anti-IgG2a 680	LI-COR 926-68051	–	1:10000
Goat anti-mouse 488	Invitrogen A32723	1:1000 to 1:2000	–
Donkey anti-rabbit 568	Invitrogen A10042	1:1000	–
Donkey anti-sheep 568	Invitrogen A21099	1:1000	–
Donkey anti-mouse 568	Invitrogen A10037	1:1000	–
Goat anti-rabbit 488	Invitrogen A11008	1:1000	–
Goat anti-chicken 647	Invitrogen A21449	1:1000	

Quantitative PCR (qPCR)

Cells were lysed and RNA extracted using the PureLink RNA Mini extraction kit (Invitrogen 12183018A) according to the manufacturer's instructions, including the following optional steps. Cells were lysed in a 6-well dish using lysis buffer with the addition of 1% (v/v) 2-mercaptoethanol. The lysate was homogenised via 10 aspirations through a 21-gauge needle attached to a 1 mL syringe. RNA was eluted in 12 μ L RNase-free water. Extracted RNA was reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA kit (Thermo Fisher, 4368814). Up to 2 μ g of RNA was used in each conversion, according to the manufacturer's instructions. The reaction was performed at 37°C in a thermocycler for 30 min before inactivation at 95°C for 5 min and dilution of cDNA to 25 ng/ μ L.

DNA amplification was performed using TaqMan FAM labelled probes against GAPDH (Hs02786624_g1) and VGLUT1 (a.k.a. SLC17A7; Hs00220404_m1). 20 μ L reactions were prepared, containing 1 $\times$ TaqMan master mix, 1 $\times$ TaqMan assay probe, 100 ng cDNA and RNase-free water. A sample where no RT enzyme was added during cDNA preparation was used to assess genomic DNA contamination. Reactions were performed in white-bottomed 96-well plates on a CFX96 real-time thermal cycler (Bio-Rad) with the following thermal cycling protocol: 10 min at 95°C then 40 cycles of melting at 95°C for 15 s followed by annealing and extension at 60°C for 1 min, with fluorescence measurement of all wells. The experiment was performed on three biological replicates, each measured in technical triplicate. Expression changes were measured using the $\Delta\Delta$ Ct method [61] and significance of log₂ fold-change was assessed via two-tailed T test using Prism 7 (GraphPad).

Viruses

Wild-type HSV-1 strain KOS (WT HSV-1) was derived from a bacterial artificial chromosome (BAC) encoding the KOS genome [62], as was HSV-1 with EFYP-tagged ICP0 and mCherry-tagged gC (timestamp HSV-1) [63]. A mutant timestamp HSV-1 lacking expression of pUL21 (Δ pUL21 timestamp HSV-1) was generated by two-step red recombination [62] of the timestamp

BAC, introducing three stop codons into the pUL21 gene as described in [64]. Initial transfection of purified BAC DNA and pGS403 (encoding Cre recombinase) and all subsequent propagation was performed in complementing U2OS pUL21-BirA*-HA cells to minimise the chance of virus adaptation [60]. HSV-1 strain 17 was from Stacey Efstathiou (University of Cambridge, UK) and HSV-1 SC16 was from Tony Minson (University of Cambridge, UK).

Virus stocks were grown by infection of U2OS pUL21-BirA*-HA cells (Δ pUL21 timestamp HSV-1) or Vero cells (all others) at low (0.01) multiplicity of infection (MOI) for 3–5 days, until widespread cytopathic effect was evident. For timestamp viruses, cells were scraped into medium, freeze-thawed and sonicated at 50% amplitude for 45 seconds in a cuphorn sonicator before being clarified by centrifugation at 3,200×g for 5 min in a benchtop centrifuge. For all other viruses, the culture medium was supplemented with 0.5 M NaCl and 100 µg/mL dextran sulfate (7–20 kDa; SigmaAldrich 51227). The following day the supernatant was harvested, filtered through a 0.8 µm cellulose nitrate membrane (Nalgene 450-0080), virions were pelleted by centrifugation at 17,000 rpm in a Type 19 rotor for 45 min at 4°C, and viruses were resuspended in PBS with 10% (v/v) glycerol. For all, virus stocks were aliquoted and stored at -70°C until required.

Virus titration

Samples serially diluted in 500 µL complete DMEM were used to infect 6-well plates containing confluent monolayers of Vero cells for 1 h at 37°C before being overlaid with 2 mL complete DMEM containing 0.3% high viscosity carboxymethyl cellulose (CMC) and 0.3% low viscosity CMC. After 3 days, cells were fixed with 3.7% (v/v) formal saline for 20 min before either being imaged using an Incucyte SX5 (Sartorius) with a 20× long working distance objective (NA 0.45) for fluorescent (timestamp) viruses or stained with 0.1% toluidine blue. In both cases plaques were counted manually.

Virus infection

For neuronal infections, day 3 i3Neurones were seeded at specified densities and allowed to mature. After maturation (day 14), conditioned medium was reserved, i3Neurones were washed with PBS and then infected with the indicated viruses in fresh CN medium at the relevant MOI. The time of virus inoculation was assigned 0 h post infection (hpi). Inoculation volumes for neurones per well were as follows: 100 µL for 96-well plates, 300 µL for 24-well plates, 600 µL for 6-well plates. Inoculated neurones were placed on a rocking platform at 37°C in a humidified 5% CO₂ atmosphere. After 1 or 2 h incubation (as indicated), the inoculum was removed, cells were gently washed twice with PBS (unless stated otherwise), and washed cells were overlaid with a 1:1 mix of fresh and conditioned CN medium that had been clarified by centrifugation. Final volumes of overlay per well were as follows: 200 µL for 96-well plates, 1 mL for 24-well plates and 2.5 mL for 6-well plates. Vero cells were infected as above but using complete DMEM in place of CN medium.

Immunoblotting

Day 3 i3Neurones were seeded at 2×10⁶ cells per well in a PLO-treated 6-well plate and allowed to mature. Where indicated, day 14 cells were infected at MOI 5 for 1 h with HSV-1 strains KOS, strain 17 or SC16, or mock infected. For all infections, cells were harvested 24 hpi, and for all other samples cells were harvested at day 14. Cells were washed once with room temperature (RT) PBS and then ice-cold PBS containing 1% EDTA-free protease inhibitor cocktail (SigmaAldrich P8849) was used to detach the neurone layer from the well, fully suspending it by

quickly and repeatedly dispensing down the edge of the well. The cell suspension was transferred to a microcentrifuge tube and pelleted (5000 × g, 5 min, 4°C) to remove the supernatant before lysis for 5 min using ice-cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% EDTA-free protease inhibitor cocktail) and centrifugation (9000 × g, 10 min, 4°C) to remove debris. Undifferentiated i3Neurone iPSCs were grown to ~80% confluence in Matrigel-coated 6-well plates before being washed with PBS, lysed in situ, transferred to microcentrifuge tubes and the lysate clarified as above. For both, protein concentrations were analysed by BCA assay (Pierce) and normalised before samples were boiled (95°C, 5 min) in Laemmli sample buffer and separated by SDS-PAGE. Separated proteins were transferred to Protran 0.45 µm nitrocellulose membranes (Cytiva) using the Mini-PROTEAN system (Bio-Rad). Membranes were blocked using Tris-buffered saline (TBS; 50 mM Tris pH 7.6, 150 mM NaCl) supplemented with 5% (w/v) skim milk powder. Primary and secondary antibody incubations were performed in TBS supplemented with 0.1% TWEEN (TBS-T) and 5% (w/v) skim milk powder. Immunoblots were imaged using an Odyssey CLx (LI-COR) and analysed using Image Studio Lite (LI-COR).

Immunocytochemistry

For confocal imaging, 13 mm #1.5 borosilicate glass coverslips were etched with 1 M nitric acid for 24 h before washing with ethanol and sterile PBS then coated with 100 µg/ml PLO for 24 h. Day 3 i3Neurons were seeded on prepared coverslips at a density of 1×10^5 cells per coverslip and were allowed to mature. After maturation (day 14), cells were washed with PBS and fixed with the 4% (v/v) EM-grade formaldehyde (Polysciences) in a 250 mM HEPES pH 7.5 buffer on ice for 10 min followed by an 8% (v/v) formaldehyde solution in 250 mM HEPES pH 7.5 buffer at room temperature (RT) for 20 min, unless otherwise stated. Fixed i3Neurons were washed thrice with PBS and permeabilised at RT on a rocking platform using PBS supplemented with 0.2% Triton X-100 for 5 min or 0.1% saponin for 15 min. Cells were blocked for 30 min at RT using blocking buffer comprising PBS supplemented with 5% (v/v) FBS alone (Triton X-100 permeabilisation) or 5% (v/v) FBS plus 0.01% saponin (saponin permeabilisation) and incubated with primary antibody in blocking buffer for 2 hr at RT. Coverslips were washed in blocking buffer, incubated with secondary antibodies in blocking buffer for 1 hr at RT in the dark, then washed in blocking buffer, PBS and then MQW. Cells were mounted on microscope slides using Mowiol 4-88 (Merck) supplemented with 200 nM 4',6-diamidino-2-phenylindole (DAPI) and left to dry overnight before being stored at 4°C. Slides were imaged using an EVOS M5000 (Invitrogen) using a 20× plan fluorite long working distance objective (NA 0.45) or a Zeiss LSM700 confocal laser scanning microscopy system mounted on an AxioObserver.Z1 inverted microscope with a 64× plan apochromat objective (NA 1.4).

For monitoring percentage infection, cells grown in PLO-treated culture vessels were fixed in situ using 4% then 8% formaldehyde in HEPES buffer and stained for ICP4 as detailed above. Immunostained cells were overlaid with propidium iodide (2.5 µg/mL in PBS + 0.02% sodium azide) to stain the nuclei and were imaged via phase contrast and fluorescence using an Incucyte SX5 (Sartorius) with a 20× long working distance objective (NA 0.45). Images were quantified using the Basic Analyser algorithm in the Incucyte analysis software (Sartorius).

For all, figures were generated using ImageJ [65, 66].

265 *Inoculation condition optimisation*

266 Day 3 i3Neurones were seeded at 3×10^5 cells per well in a PLO-treated 24-well plate and
267 allowed to mature. Infection proceeded as described above with changes to the inoculation
268 conditions as follows: cells were inoculated for 60, 90 or 120 min being rocked either manually
269 every 15 min or automatically at a low speed on a rocking platform. The inoculum was removed
270 and cells gently washed with PBS twice (unless stated otherwise), overlayed with a 1:1 mix of
271 fresh and clarified conditioned CN medium. Cells were fixed at 16 hpi and percentage infection
272 was monitored by immunocytochemistry using 4 to 8% formaldehyde in HEPES buffer for
273 fixation and Triton X-100 for permeabilisation as described above. Data were analysed using a
274 two-way ANOVA and significance of differences to 60 min sample was assessed using Sidak's
275 test in Prism 7 (GraphPad). The equivalency of variance across all data points at MOI 5 for the
276 automatic versus manual rocking was assessed using Levine's test [67] in Prism 7 (GraphPad).

277 *Percentage of infected i3Neurones at different MOIs*

278 Day 3 i3Neurones were seeded at 3×10^5 cells per well in a PLO-treated 24-well plate and
279 allowed to mature. At day 13, Vero cells were seeded separately at 1×10^5 cells per well. The
280 following day, both i3Neurones and Vero cells were infected with a 2-fold serial dilution of WT
281 HSV-1, with MOIs ranging from 10 to 0.0098. After 2 h the inoculum was removed and the cells
282 gently washed twice with PBS (unless stated otherwise) before being overlayed with a 1:1 mix of
283 fresh and clarified conditioned media. Cells were fixed at 16 hpi and percentage infection was
284 monitored by immunocytochemistry as described above.

285 *Neurone survival following infection*

286 Day 3 i3Neurones were seeded at 3×10^5 cells per well in a PLO-treated 24-well plate and
287 allowed to mature before being infected at MOI 5 for 1 h with HSV-1 strains KOS, strain 17 or
288 SC16, or mock infected. Cells were washed twice with PBS, and then overlayed with a 1:1 mix of
289 fresh and clarified conditioned CN medium supplemented with 2.5 $\mu\text{g/mL}$ propidium iodide.
290 Cell viability, defined as exclusion of the propidium iodide, was monitored by capturing phase
291 contrast and orange fluorescence images every 6 h using an Incucyte SX5 (Sartorius). Images
292 were analysed using the Basic Analyser algorithm in the Incucyte analysis software (Sartorius).
293 Half the volume of CN medium, supplemented with fresh 2.5 $\mu\text{g/mL}$ propidium iodide, was
294 replaced every 3 days.

295 *Inoculum inactivation*

296 Day 3 i3Neurones were seeded at 3×10^5 cells per well in a PLO-treated 24-well plate and
297 allowed to mature. The i3Neurones were infected at MOI 5 for 1 hr before the inoculum was
298 removed and cells were either: washed thrice immediately with PBS; incubated with 25 $\mu\text{g/mL}$
299 LP2 for 15 min at 37°C before three PBS washes; or washed once with citric acid (40 mM citric
300 acid pH 3.0, 135 mM NaCl, 10 mM KCl) for 1 min before three PBS washes. Cells were overlayed
301 with a 1:1 mix of fresh and clarified conditioned CN medium. At 3 and 24 hpi, neurones were
302 harvested by thrice freezing the plate at -70°C and then thawing. Lysed cells were scraped into
303 the medium and the virus concentration was assessed by plaque assay. Data were analysed
304 using a one-way ANOVA and significance was assessed using Tukey's test in Prism 7
305 (GraphPad).

306 *Fixative Condition Tests*

307 Day 3 i3Neurones were seeded at 1×10^5 cells per well on prepared coverslips in 24-well plates
308 and allowed to mature. The i3Neurones were infected with WT HSV-1 at MOI 3. At 16 hpi,
309 neurones were washed with PBS and fixed using one of the following four treatments: 4% (v/v)
310 EM-grade formaldehyde in a 250 mM HEPES pH 7.5 on ice for 10 min then an 8% (v/v)
311 formaldehyde solution in 250 mM HEPES pH 7.5 at RT for 20 min; cytoskeletal fixing buffer
312 (300 mM NaCl, 10 mM EDTA, 10 mM glucose, 10 mM $MgCl_2$, 20 mM PIPES pH 6.8, 2% sucrose,
313 4% formaldehyde) on ice for 15 min; 100% methanol on ice for 10 min; or glyoxal buffer pH 4
314 (3% (v/v) glyoxal, 20% (v/v) ethanol, 0.75% acetic acid, pH adjusted with NaOH) on ice for 30
315 min followed by a further 30 min at RT. Fixed neurones were washed, permeabilised, stained
316 and imaged as described above.

317 *HSV-1 spread assay*

318 Day 3 i3Neurones were seeded at 3×10^5 cells per well in a PLO-treated 24-well plate and
319 allowed to mature before being infected at MOI 0.1 for 2 h with timestamp viruses, washed
320 twice with PBS, and then overlaid with a 1:1 mix of fresh and clarified conditioned CN medium
321 supplemented with 25 μ g/mL LP2 antibody. Virus spread was monitored by live-cell
322 fluorescence imaging using an Incucyte SX5 (Sartorius), recording phase contrast images, green
323 fluorescence and orange fluorescence every 3 h. Data were analysed using the Basic Analyser
324 algorithm in the Incucyte analysis software (Sartorius). Half the volume of CN medium was
325 replaced every 3 days.

326 **Results**

327 *Optimisation of the i3Neurone differentiation protocol for infection studies*

328 The protocols for iPSC culture and i3Neurone differentiation were adapted from [49] to increase
329 the efficiency of differentiation and promote survival during maturation (Fig. 1a). Specifically,
330 duration of the Accutase dissociation was decreased to reduce cell death. The plating
331 procedure was modified by seeding cells in half the final culture volume of medium and
332 incubating the cells on the plate for 15 min at RT before adding the remaining medium. This
333 ensured neurones evenly coated the well and reduced clumping of neurone cell bodies. To
334 ensure iPSCs had correctly differentiated into glutamatergic neurones following these
335 adaptations, immunoblotting (Fig. 1b), qPCR (Fig. 1c) and immunofluorescence microscopy
336 (Fig. 1d) of cultured iPSCs and i3Neurones was performed. Immunoblotting confirmed the loss
337 of pluripotency marker OCT4 and the gain of neuronal markers TAU and β III tubulin following
338 differentiation. Gene expression of VGLUT1 (a.k.a. SLC17A7), a vesicular glutamate transporter
339 that is widely used as a marker for glutamatergic signalling in neurones [48, 68], is significantly
340 increased in differentiated i3Neurones compared to undifferentiated iPSCs. The presence of
341 extensive neurites and neuronal cytoskeletal proteins MAP2 and TAU (Fig. 1d) also confirmed
342 successful differentiation.

343 *Optimisation of i3Neurone infection protocol*

344 Initial infection tests suffered from technical issues including cell death, cell layers peeling off
345 the plate and lower levels of infection than expected given the titre of virus inoculum. Several
346 strategies were employed to combat these issues, testing different inoculation conditions. The
347 first optimisation was to increase the volume of medium used for virus inoculation, as
348 otherwise i3Neurones dried out and died during the inoculation step (Fig. S1a). Using

approximately 30–50% of the final overlay volume for inoculation prevented such cell death. Secondly, to prevent peeling of the neurone layer (Fig. S1b) it was important to pipette liquids dropwise directly onto the cell layer rather than pipetting liquid onto the walls of the culture vessel [49].

To test if inoculation conditions could be optimised to increase the proportion of cells infected, neurones were infected at an MOI of either 1 or 5 via incubation with inoculum for 60, 90 or 120 min, either with continual rocking on a rocking platform or with manual rocking every 15 min (Fig. 2a). Cells were fixed at 16 hpi and stained for ICP4, an immediate-early viral protein that localises predominantly to the nucleus [69, 70], plus propidium iodide to visualise nuclear DNA. Cells were imaged via automated microscopy to determine the percentage of infected cells (Fig. 2b). Automatic or manual rocking did not make a statistically significant difference to efficiency of infection at either MOI (two-way ANOVA of three independent experiments, $p = 0.718$ [MOI 1] or 0.280 [MOI 5]). At MOI 1 there was a significant increase in infection efficiency with increased duration of inoculation (Fig. 2a), but even after 2 h incubation with inoculum the proportion of infected cells remained below the theoretical maximum of 63.2% expected if infections occurred randomly. Extending the inoculum incubation time at MOI 5 did not alter the efficiency of infection, but use of an automated rocker resulted in significantly less variability of infection level across the replicates and time points (Levine's test, $p = 0.0346$).

To further investigate the efficiency of infection, a 2-fold dilution series of WT HSV-1 (from MOI 10 to 0.01) was used to inoculate i3Neurones and Vero cells in parallel (Fig. 2c). Below MOI 5 the proportion of infected cells was consistently lower than theoretically expected. Interestingly, a higher proportion of Vero cells were infected than expected, suggesting that virus titration via plaque assay may systematically underestimate the infectious titres. However, this greater-than-expected infection could also, in part, be ascribed to expression of the immediate-early gene ICP4 in cells that did not progress to full lytic infection [71, 72]. At high MOI, automated measurement of infection in Vero cells suggested that >100% of cells were infected. This arose due to the non-homogenous distribution of ICP4 staining in nuclei, with some infected Vero cell nuclei being counted twice by the Incucyte software (Fig. S2). Such double-counting may have also contributed to the greater-than-expected level of infection observed for Vero cells at different MOIs. Since double-counting of infected cells was not observed for i3Neurones, this phenomenon was not investigated further.

For temporally-resolved experiments like high MOI (single-step) growth curves that require a synchronous infection, it is necessary to inactivate any input virus particles that have not entered cells after a fixed time. This inactivation is often achieved via low pH treatment [73, 74]. Because the neurones may be more sensitive to chemical treatment than other cell lines, different methods were used to assess their inactivation efficiency whilst preserving cell viability. Differentiated i3Neurones were infected at MOI 5 using an automated plate rocker. After 1 h the inoculum was removed and cells were washed with citric acid, incubated with a potent neutralising antibody (LP2), or washed with PBS. The cultures were harvested at 3 and 24 hpi to assess both inoculum inactivation and subsequent virus production, which would decrease dramatically if cell viability was affected (Fig. 2d). The citric acid wash was the most effective treatment for inactivation, reducing the viral titre to below detection in some replicates. The antibody incubation was marginally more effective than PBS washing alone at removing input virus, but both were inferior to a citrate wash. There was no significant difference in virus yield at 24 hpi between any of the three conditions, demonstrating that none of these

protocols adversely affected neurone viability. Based on these optimisations, refined protocols for low- and high-MOI infection of i3Neurones are summarised in Fig. 2e.

During the optimisation of the infection protocol, it was noted that neurones seemed highly tolerant of infection, with reduced morphology changes and prolonged survival compared to other cell types like Vero. The survival of i3Neurones following synchronous high MOI infection was thus investigated. Neurones were infected at MOI 5 with HSV-1 strains KOS, strain 17 or SC16, or mock-infected. Neurones were then incubated in the presence of propidium iodide, a DNA stain excluded from live cells, and imaged every 6 h via automated microscopy. By 5 days post-infection the neurones displayed morphology changes, with changes in the appearance of the soma and, in the case of KOS, clustering of the soma that is potentially indicative of syncytium formation. However, the neurones remained alive for upwards of 8 (KOS) or 10 (strain 17 and SC16) days, with visibly axonal degradation occurring close to the time of cell death (Fig. 3).

Optimised fixation and permeabilization of i3Neurones for immunocytochemistry

Neurites often became visibly damaged during the fixation and staining procedures used for preliminary infection quantification experiments, presumably due to their delicate nature. Different fixation conditions were thus tested for i3Neurones grown on coverslips that had been acid-etched to improve neurone adhesion and then infected at MOI 3 with HSV-1 [75]. Two conditions commonly used for immunocytochemistry of epithelial cells were tested (4% followed by 8% formaldehyde in 250 mM HEPES pH 7.4, or 100% methanol), as were two identified in the literature as being more effective for neurones or for preserving cytoskeletal structures (3% glyoxal pH 4.0 in 20% ethanol, or 4% formaldehyde in a cytoskeletal preservation solution)[76, 77]. Two different permeabilisation solutions were trialled, either 0.2% Triton X-100 for 5 min or 0.1% saponin for 15 min. i3Neurones were stained for the neuronal cytoskeletal protein β III tubulin, the *trans*-Golgi network protein TGN46, the nuclear marker of infection ICP4, and for DNA using DAPI. The blocking and staining protocols used for each fixation/permeabilisation condition was identical, except for the inclusion of 0.01% saponin in the blocking buffer for cells permeabilised with saponin. Wide-field microscopy of coverslips fixed using the different protocols and permeabilised with Triton X-100 or saponin are shown in Figs S3 and S4, respectively.

Fixation using 4% then 8% formaldehyde in 250 mM HEPES yielded the best preservation of cells morphology, with excellent preservation of neurites. Triton permeabilization yielded visibly brighter signal for TGN46 and similar staining for the other markers, both in wide field (Figs S3 and S4) and confocal (Fig. 4) microscopy. Acquiring confocal Z-stacks (7–14 nm) proved the most reliable method for imaging both the thin neurites and the thicker cell bodies. It is notable that at 16 hpi there is no apparent change in overall cell morphology (contrast Fig. 1c and Fig. 4).

i3Neurones as a model for HSV-1 lytic infection of cortical neurones

Having optimised the infection procedure, the utility of i3Neurones for monitoring HSV-1 spread was assessed. Plaque assays, which monitor the spread of virus to adjacent cells following infection of a single cell, are a well-established technique for measuring HSV-1 cell-to-cell spread in cells of the periphery like fibroblasts [30, 78]. However, the sparsity of neuronal cell bodies and potential for long-distance spread via intracellular transport of virions along neurites confounds the use of plaque assays to measure HSV-1 spread in i3Neurones. Therefore, virus neurone-to-neurone spread was monitored by infecting i3Neurones at low MOI (0.1) with HSV-1

strain KOS expressing the immediate-early protein ICP0 tagged with EYFP and the late protein gC tagged with mCherry ('timestamp HSV-1') [63]. Neutralising antibody (LP2) was included in the culture medium to inhibit cell-free spread and the spread of fluorescence, a proxy for infection spread, was monitored every 3 h by automated microscopy. No signal was visible for EYFP-ICP0 on the automated Incucyte SX5 microscope, despite the presence of a robust EYFP signal at 20 hours post-infection in i3Neurons infected at MOI 0.1 with Timestamp HSV-1 (Fig. S5). However, a robust gC-mCherry signal was evident and this signal spread throughout the culture over the course of 96 h (Fig. 5a), confirming productive neurone-to-neurone HSV-1 spread. The infection appeared to spread most rapidly between the soma of adjacent cells but by 48 hpi infection was also evident in the soma of distant cells, consistent with spread of the infection via axonal transport of newly produced HSV-1 virions.

Many proteins present within the tegument layer of HSV-1 are known to contribute to efficient cell-to-cell spread in fibroblasts or keratinocytes [64, 79]. However, the contributions these proteins make to neurone-to-neurone spread is less clear. The role of tegument protein pUL21 is of particular interest, as its effect upon virus spread is known to vary by HSV strain and by cell type [80]. To assess its contribution to neurone-to-neurone spread, a mutant virus lacking expression of pUL21 was generated in the timestamp background (timestamp Δ pUL21, Fig. S6). The spread of timestamp Δ pUL21 infection was monitored via automated microscopy following low MOI (0.1) infection of i3Neurons (Fig. 5a). The spread of timestamp Δ pUL21 was substantially delayed when compared to the parental timestamp virus (Fig. 5b). However, there was still evidence of spread to soma of neurones distal to the initial site of infection, suggesting that transport of virions along neurites had not been completely impaired. Taken together, this experiment demonstrates how i3Neurons can be combined with fluorescently tagged HSV-1 to measure the effects of HSV-1 proteins on viral neurone-to-neurone spread.

In addition to monitoring virus spread, it is often desirable to monitor the abundance of cellular or viral proteins in synchronous populations of infected cells. While immunoblotting is a convenient technique for monitoring protein abundance, it can be difficult to obtain enough infected-cell lysate for immunoblotting when working with organoids or primary neurones. The scalability of i3Neurons [49], combined with the ability to perform efficient synchronous infection (Fig. 2), overcomes this limitation. To assess the feasibility of using immunoblots to monitor changes in host protein abundance, single wells of a 6-well dish containing 2×10^6 i3Neurons were synchronously infected (MOI 5) with HSV-1 strains KOS, strain 17 and SC16. At 24 hpi cells were harvested, lysed and subjected to immunoblot analysis. For all three strains the viral capsid protein VP5 could be detected, confirming successful infection and late gene expression (Fig. 6). Additionally, compared to the mock-infected sample, all three infected lysates showed lower abundance of the cellular protein GOPC, a known target of HSV pUL56-mediated degradation [30]. This confirms that the i3Neurone system is suitable for biochemical analysis of HSV-1 neuronal infection.

Discussion

Here we present optimised protocols for the differentiation of human iPSC-derived cortical glutamatergic neurones (i3Neurons) and their infection with HSV-1. The i3Neurone system is highly scalable, allowing production of $>10^7$ differentiated neurones with ease, and these neurones can be synchronously infected with high ($>90\%$) efficiency (Fig. 2). These neurones survive for upwards of 8 days following infection (Fig. 3), consistent with previous reports of sympathetic mouse neurones surviving for up to 30 days following lytic infection with HSV-1 [81]. We show that i3Neurons are suitable for biochemical analysis of lytic HSV-1 infection

(Fig. 6) and i3Neurones thus show strong potential for use in high resolution infection proteomics analysis [29, 30]. We have previously shown that i3Neurones can be infected with Zika virus [82], human astroviruses [83] and human enteroviruses [84]. i3Neurones thus represent a promising platform for advanced biochemical analysis of many neurotropic virus infections.

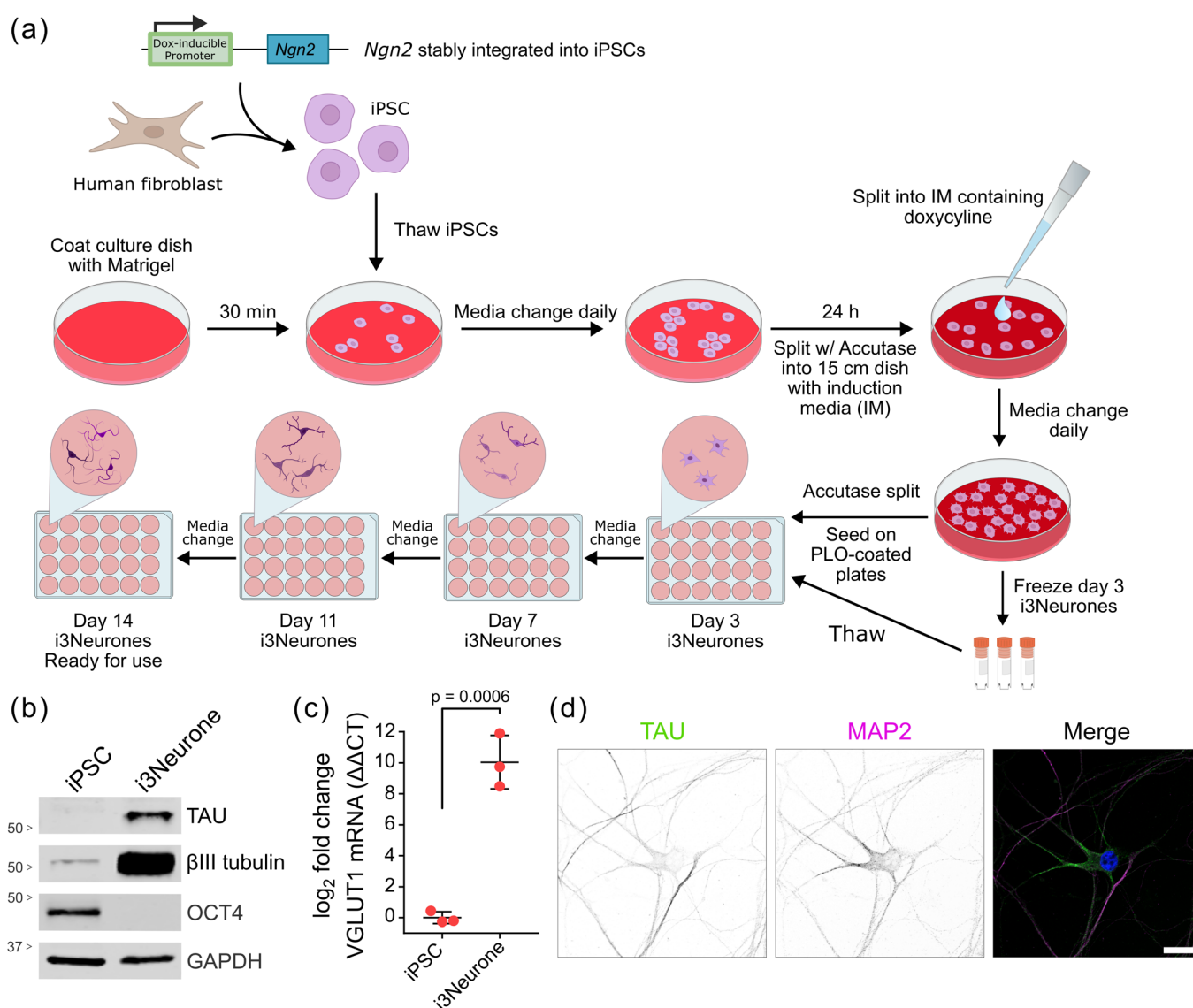
In addition to biochemical analyses, the reproducibility of i3Neurone differentiation [48] and their amenability to gene overexpression [53] or knockdown via the integrated dead Cas9 [50, 53, 54] make them a powerful platform for functional analysis. We show here that i3Neurones can be combined with fluorescent virus strains of HSV-1 to monitor neurone-to-neurone spread of HSV-1. While we observed a signal for ICP0-EYFP in i3Neurones when imaged using a wide-field microscope (Fig. S5), consistent with prior studies using timestamp HSV-1 [63, 85], we could not visualise ICP0-EYFP using the Incucyte SX5 automated microscope. This is likely a consequence of the suboptimal matching of the excitation (453–485 nm) and emission (494–533 nm) filters on our automated microscope to the EYFP fluorophore (peak excitation 515 nm and emission 530 nm). However, we note that the 3' untranslated region of the ICP0-encoding *RL2* gene in timestamp HSV-1 retains the previously-identified miRNA-138 binding site [86]. miRNA-138 is highly expressed in multiple neuronal cell types [87] and its binding of *RL2* mRNA suppresses ICP0 expression [86]. It is thus likely that HSV-1 infected i3Neurones express lower levels of ICP0 than equivalently infected fibroblasts or keratinocytes, although we did not test this directly.

In the presence of neutralising antibody, we show that HSV-1 strain KOS spreads to the soma of neurones far from the initial site of infection within 48 hpi, consistent with intracellular transport of virions along neurites. It is unclear whether this spread represents virus particles budding from the soma of an infected cell, entering a neurite and undergoing retrograde transport to the nucleus, or whether it represents anterograde transport of newly assembled virions to neurite termini where they then bud to infect other neurones. Since HSV-1 strain KOS lacks a functional pUS9 protein [88], known to be important for both anterograde axonal transport and virus assembly at axon termini [89], it seems likely that the observed long-distance spread represents retrograde transport following infection of neurites. This could be confirmed in future studies using directional infection of soma or neurites in compartmentalised culture systems [90]. Given the absence of pUS9, it is potentially surprising that i3Neurones infected with HSV-1 strain KOS die more rapidly than those infected with SC16 or strain 17 (Fig. 3). We note that the soma of i3Neurones infected with strain KOS are more extensively clustered, suggesting that a cytopathic effect causes the accelerated cell death. However, the precise mechanisms that drive clustering of soma and accelerated cell death remain a topic for future study.

Differentiated i3Neurones represent cortical glutamatergic neurones of the cerebral cortex and thus provide a model system relevant to studying HSE at the cellular level. The protocols for lytic infection of i3Neurones described here will enable future studies to analyse the effects of lytic HSV-1 infection on the brain. i3Neurones use doxycycline-induced NGN2 expression in human iPSCs to determine differentiation into a cortical glutamatergic subtype, whereas other recently developed iPSC systems utilise doxycycline-induced expression of NGN3 for differentiation into sensory neurones for studying HSV-1 latency [43, 91, 92]. Both systems offer an iPSC- derived method to model human neuronal infections by HSV-1 and both support lytic replication, producing similar viral titres following infection (Fig. 2D) [44, 46]. The differentiation protocol requires approximately 18–21 days for these sensory neurones (iNGN3) to reach maturity [46], similar to the time required for i3Neurones to become electrically mature [48, 50].

Differentiation of iNGN3 neurones comprises three distinct stages, versus two for i3Neurones. Both systems require doxycycline treatment to initiate differentiation, but whereas i3Neurones can then be plated in CN medium for maturation, iNGN3 neurones require a further differentiation stage involving the addition of five small-molecule inhibitors (LDN193189, SB431542, DAPT, SU5402, and CHIR99021). The final maturation stage for both cell lines is similar, but iNGN3 neurones require two additional growth factors (GDNF and β -NGF) [46, 49]. NGN2 expression in i3Neurones results in complete differentiation, with surviving cells committing to a post-mitotic neuronal state, while mitomycin C or 5-fluorodeoxyuridine treatment is necessary during maturation of iNGN3 neurones to remove undifferentiated mitotic cells [46]. NGN3-mediated differentiation with these inhibitors yields a mixed population of sensory neurone subtypes that represent the natural sites of HSV-1 latency, whilst i3Neurones solely represent cortical glutamatergic neurones [46, 48]. The capacity of i3Neurones to support latency has yet to be tested, and although detection of HSV-1 DNA in the brain without clinical signs of HSE is well documented [93–95], only one report to date has identified latency-associated transcripts in the human cortex [96]. iNGN3 sensory neurones thus constitute a more relevant model for studying HSV latency, but establishing latency in the i3Neurone cortical system remains an intriguing prospect for future research should further evidence emerge for HSE resulting from reactivation of latent or quiescent HSV-1 within infected cortical neurones.

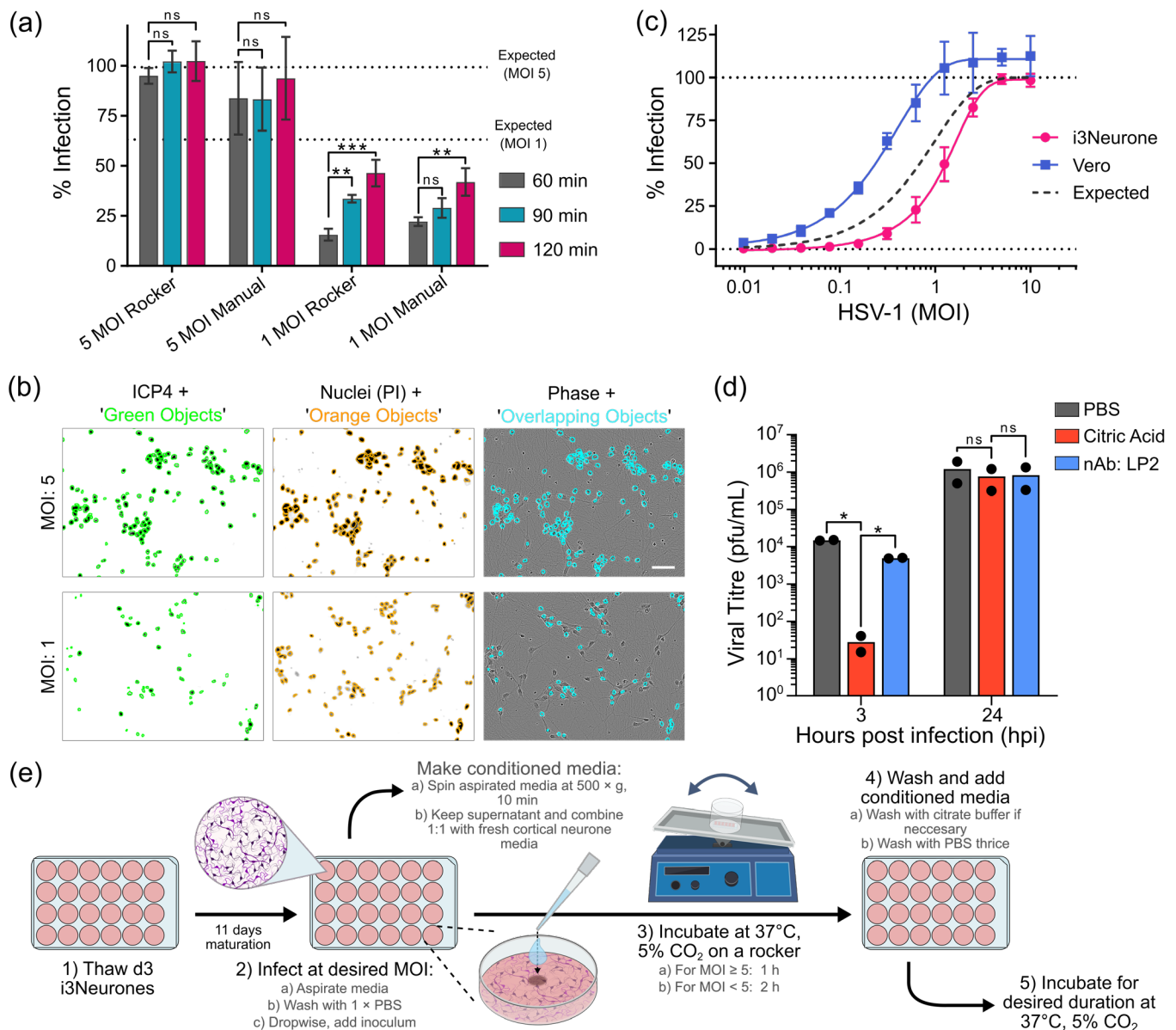
In summary, using HSV-1 as a model we have demonstrated the i3Neurone system to be a robust tool for measuring the replication and spread of viruses in cortical neurones. We anticipate that optimised neurone culture, infection and analysis protocols presented here will accelerate research into a broad range of clinically important neurotropic infections.



553

554 **Fig. 1.** Differentiation of human iPSCs into cortical glutamatergic neurones (i3Neurons). (a)
 555 Schematic of the differentiation procedure. PLO, poly-L-ornithine; *Ngn2*, Neurogenin 2. (b)
 556 Validation of iPSC differentiation into neurones. Lysates of iPSCs and i3Neurons were
 557 immunoblotted for pluripotency marker OCT4 and neuronal markers TAU and β III-tubulin.
 558 GAPDH is a loading control. (c) Validation of differentiation into a glutamatergic subtype.
 559 Expression of VGLUT1 in i3Neurons was assessed by qPCR and is shown as log₂ fold-change
 560 relative to iPSCs. Mean $\pm$ SD from 3 biological replicates, each performed in technical triplicate,
 561 is shown. Two-tailed T test $p = 0.0006$. (d) Confocal microscopy of differentiated i3Neurons,
 562 showing neurone-like morphology. Neuronal markers TAU (green) and MAP2 (magenta) are
 563 shown, and the merge image includes DAPI (blue). 20 μ m scale bar.

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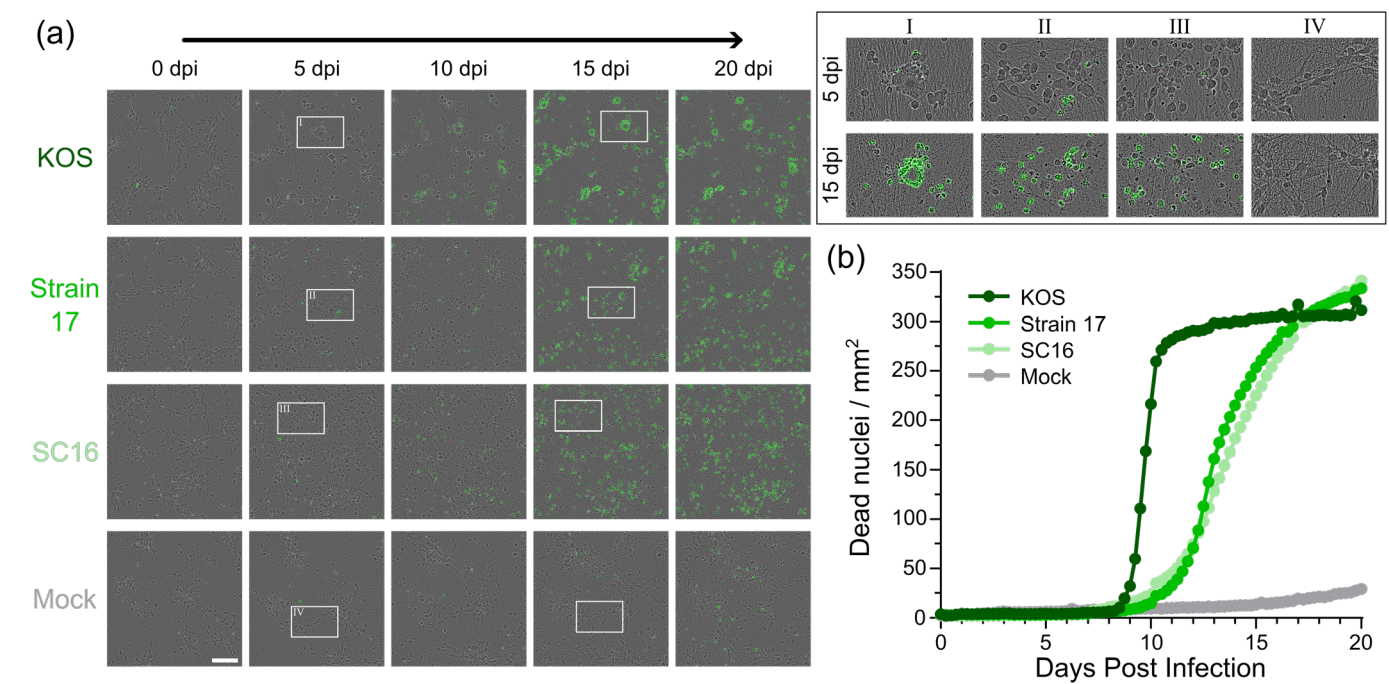


565

566 **Fig. 2.** Optimisation of i3Neurone infection by HSV-1. (a) i3Neurones were infected at MOI 5 or 1
567 and incubated for the listed duration with manual or automated rocking. Percent of infected
568 cells as determined by automated microscopy (mean $\pm$ SD from three independent
569 experiments) is shown. Two-way ANOVA (automated or manual rocker versus time) confirmed
570 no significant effect of rocking method but a significant effect of time for MOI 1 but not MOI 5.
571 ns, no significance; **, p < 0.005; *** p < 0.001. (b) Representative automated microscopy
572 images of infected i3Neurones. Objects where ICP4 (green, left) and propidium iodide (orange,
573 middle) signals overlap (cyan, right) are counted as infected cells, expressed as a percentage of
574 total propidium iodide objects (total cells). 50 μ m scale bar. (c) Percentage of i3Neurones and
575 Vero cells infected at different MOI (mean $\pm$ SD from three independent experiments). The
576 percentage expected if infections occur randomly is shown. (d) Optimisation of inoculum
577 inactivation. Inoculum was removed at 1 hpi (MOI 5 HSV-1) and i3Neurones were either washed
578 with PBS, incubated with neutralising antibody (nAb LP2) for 15 min, or washed with citric acid
579 pH 3.0. Cells were harvested at 3 or 24 hpi and virus titres were determined by plaque assay.
580 Mean and data points from two independent experiments, each performed in technical

581 duplicate. One-way ANOVA confirms that the citrate wash yields a significant inactivation of
582 input virus (3 hpi) but no difference in virus production at 24 hpi. ns, no significance; *, $p < 0.05$.
583 (e) Schematic diagram of the optimised workflow for infecting i3Neurones with HSV-1.

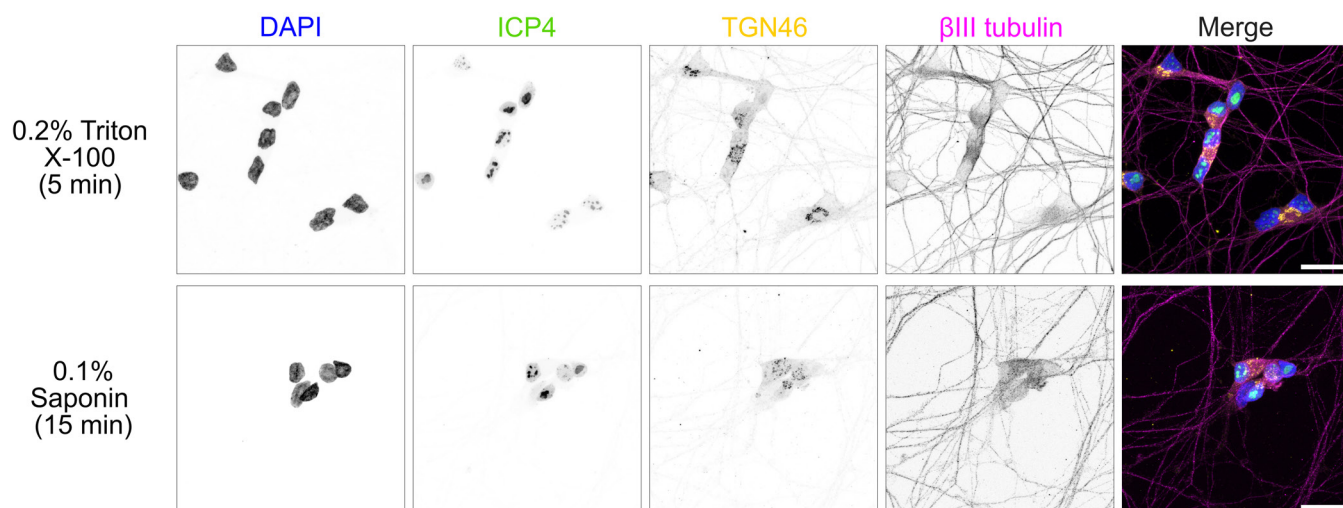
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585

586 **Fig. 3.** i3Neurones survive for over one week following HSV-1 infection. (a) Live-cell microscopy
 587 of i3Neurones infected at MOI 5 with HSV-1 strains KOS, strain 17 and SC16, at listed days post
 588 infection (dpi). Propidium iodide signal, which is excluded from live cells, is shown in green.
 589 Scale bar 100 μm . (b) Quantification of neurone survival, with cell death measured as increased
 590 number of propidium iodide positive nuclei (dead nuclei / mm^2). Data are representative of
 591 three independent experiments.

592



593

594 **Fig. 4.** Confocal microscopy of HSV-1 infected (MOI 3) i3Neurones, fixed 16 hpi using 4% then
 595 8% formaldehyde in 250 mM HEPES buffer and permeabilised using either 0.2% Triton X-100 for
 596 5 min or 0.1% Saponin for 15 min. Cells were stained for ICP4 (green), TGN46 (yellow), β III
 597 tubulin (magenta) and DNA (DAPI, blue) and maximum-intensity projections of 7 nm (Triton X-
 598 100) or 14 nm (saponin) Z stacks are shown. Triton X-100 permeabilisation yields stronger
 599 cytoskeletal (β III tubulin) and organelle (TGN46) staining. 20 μ m scale bar.

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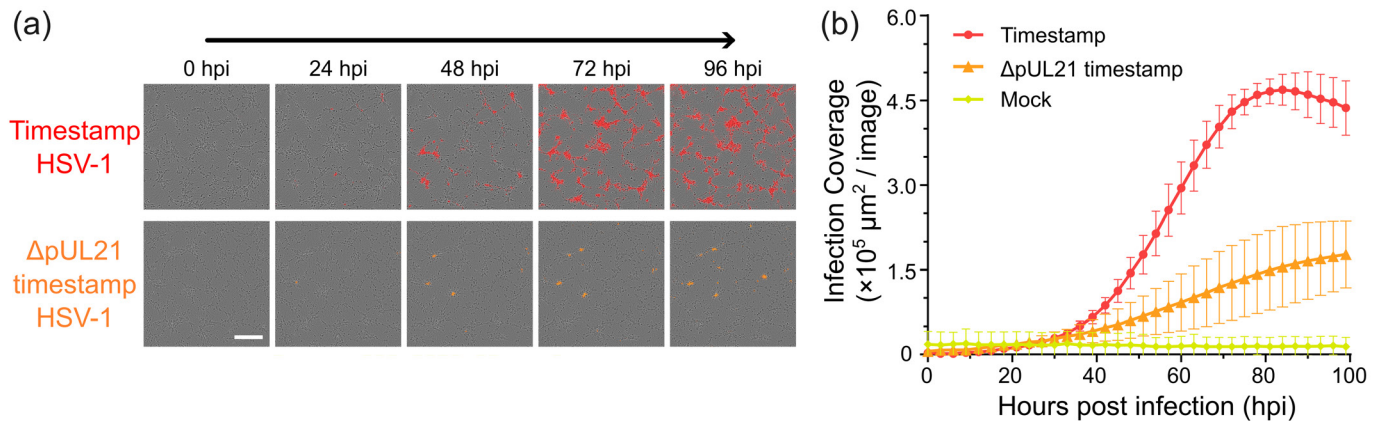


Fig. 5. HSV-1 neurone-to-neurone spread. (a) Live-cell microscopy of i3Neurons infected at low MOI (0.1) with parental or $\Delta pUL21$ timestamp HSV-1. gC-mCherry signal is shown in red or orange for timestamp and $\Delta pUL21$ timestamp HSV-1, respectively. Scale bar 200 μm . (b) Quantification of timestamp virus spread, measured as increase area of gC-mCherry fluorescence ($\mu m^2 / image$) for 4 days post infection. Mean $\pm$ SD for two independent experiments performed in technical triplicate are shown.

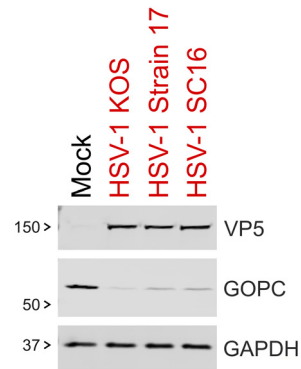


Fig. 6. Validation of viral gene expression and function in i3Neurones by immunoblot. i3Neurones were infected at MOI 5 with indicated HSV-1 strains and lysed 24 hpi. Samples were immunoblotted for infection marker VP5, the cellular protein GOPC that is a target of pUL56-mediated degradation, and the cellular loading control GAPDH.

615 **Author contributions**

616 Conceptualisation: JED, SCG; Funding Acquisition: JED, SCG; Investigation: DAN, ASN, HGB,
617 VC; Project Administration: JED, SCG; Resources: CMC, JED; Supervision: JED, SCG;
618 Visualisation: DAN; Writing – Original Draft Preparation: DAN, SCG; Writing – Review & Editing:
619 DAN, HGB, AN, JED, SCG

620 **Conflicts of interest**

621 The authors declare no competing interests.

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