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4   Physiological oxygen concentration during sympathetic primary neuron culture improves

5   neuronal health and reduces HSV-1 reactivation.

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16   Abstract

17   Herpes simplex virus-1 (HSV-1) establishes a latent infection in peripheral

18   neurons and periodically reactivates in response to a stimulus to permit transmission. *In*

19   *vitro* models using primary neurons are invaluable to studying latent infection because

20   they use bona fide neurons that have undergone differentiation and maturation *in vivo*.

21   However, culture conditions *in vitro* should remain as close to those *in vivo* as possible.

22   This is especially important when considering minimizing cell stress, as it is a well-

23   known trigger of HSV reactivation. We recently developed an HSV-1 model system that

24   requires neurons to be cultured for extended lengths of time. Therefore, we sought to

25   refine culture conditions to optimize neuronal health and minimize secondary effects on

26   latency and reactivation. Here, we demonstrate that culturing primary neurons under

27   conditions closer to physiological oxygen concentrations (5% oxygen) results in cultures

28   with features consistent with reduced stress. Furthermore, culture in these lower oxygen

29   conditions diminishes the progression to full HSV-1 reactivation despite minimal impacts

30   on latency establishment and earlier stages of HSV-1 reactivation. We anticipate that

31   our findings will be useful for the broader microbiology community as they highlight the

32   importance of considering physiological oxygen concentration in studying host-pathogen

33   interactions.

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35

36 <u>Importance</u>

37   Establishing models to investigate host-pathogen interactions requires mimicking

38 physiological conditions as closely as possible. One consideration is the oxygen

39 concentration used for *in vitro* tissue culture experiments. Standard incubators do not

40 regulate oxygen levels, exposing cells to oxygen concentrations of approximately 18%.

41 However, cells within the body are exposed to much lower oxygen concentrations, with

42 physiological oxygen concentrations in the brain being 0.55-8% oxygen. Here, we

43 describe a model for herpes simplex virus 1 (HSV-1) latent infection using neurons

44 cultured in 5% oxygen. We show that culturing neurons in more physiological oxygen

45 concentrations improves neuronal health to permit long-term studies of virus-cell

46 interactions and the impact on reactivation.

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55   Herpes simplex virus-1 (HSV-1) establishes a latent infection in peripheral

56 neurons and reactivates to cause disease. *In vitro* model systems are beneficial for

57 studying the molecular mechanisms of HSV-1 latent infection because neurons in

58 culture can be more readily manipulated. Modeling HSV-1 latency and reactivation is

59 technically challenging and requires the use of primary or differentiated neurons, which

60 are post-mitotic and therefore do not replenish. Neurons also require specialized

61 coating for adherence and supplements for survival and maturation. One challenge with

62 working with any cell type *in vitro* is keeping culture conditions as consistent as possible

63 with their *in vivo* environment.

64

65   HSV-1, like many herpesviruses, are triggered to reactivate in response to

66 cellular stress. Therefore, unwanted stress during cell culture may lead to inadvertent

67 reactivation events and confounding experimental results. We recently developed a

68 model system in which a period of 30 days is required for full entry into a latent infection

69 (1). Culturing primary neurons for this length of time can be problematic as it can be

70 challenging to keep primary neurons healthy. Therefore, we sought to optimize neuronal

71 health to enable culturing neurons for this extensive time frame and minimize

72 inadvertent stress and impacts on latency and reactivation.

73

74   Although widely accepted in scientific research, the conventional use of

75 incubators that do not regulate oxygen concentrations results in cells cultured at a non-

76 physiological concentration of approximately 18% oxygen. Neurons *in vivo* are exposed

77 to much lower oxygen concentrations of between 0.55 and 8% (2, 3). Multiple studies

78   have shown that cells cultured under more physiological oxygen conditions display

79   features consistent with better health, including increased proliferation, plating

80   efficiencies, viability, mitochondrial activity, and neurite outgrowth, as well as reduced

81   senescence and chromosomal abnormalities (4). Accordingly, we cultured sympathetic

82   neurons under more physiological oxygen concentrations, which enabled long-term

83   survival and therefore latency and reactivation studies. Here, we directly compared

84   latent infection under 5% versus atmospheric oxygen conditions to determine how

85   changing oxygen levels impacts neuronal health and HSV-1 reactivation.

86

87   In preliminary studies examining infected sympathetic neurons, we observed that

88   neurons appeared healthier in 5% oxygen (physioxic) versus atmospheric oxygen

89   conditions, with brighter, rounder soma and less axonal fragmentation (Figure 1A). To

90   quantify these changes and exclude the complicating factor of infection, we cultured

91   uninfected neurons isolated from the superior cervical ganglia (SCG) of newborn mice

92   under physioxic or atmospheric conditions for 30 days and used a previously described

93   matrix to assess neuronal health (5) (Table S1, Table S2, Figure 1B-C). Neurons under

94   physioxia demonstrated significantly reduced scores beginning at approximately 12-15

95   days post-plating. This differential in scores between physioxic and atmospheric

96   conditions remained until the conclusion of the experiment.

97

98   Culturing neurons in microfluidic devices that isolate the axons from the soma

99   compartment permits the specific treatment or infection of axons. However, this involves

100   using miniscule amounts of media, leaving neurons more susceptible to hyperoxia

101 under atmospheric conditions, and in our hands more susceptible to spontaneous

102 degeneration. Therefore, we compared spontaneous degeneration of neurons cultured

103 in microfluidic devices. At 10-days post culture, we performed immunofluorescence

104 using Beta III tubulin to visualize axons. Axons from neurons cultured under

105 atmospheric conditions demonstrated significantly more fragmentation than those

106 cultured under physioxic conditions (Figure 1D). This was quantified using a

107 degeneration index based on quantification of the ratio of fragmented axons over the

108 total axon area (6) (Figure 1E). Therefore, the health of neurons isolated from the SCG

109 is improved following incubation under physiologic conditions versus atmospheric

110 conditions independently of infection.

111

112   We next investigated the impact of oxygen concentration on HSV-1 latency and

113 reactivation. Neurons were cultured under atmospheric or physioxic conditions, and

114 infected with HSV-1 Stayput-GFP in the presence of viral DNA replication inhibitor

115 acyclovir (ACV) as previously described (1). At six days post-infection, ACV was

116 washed out, and neurons were cultured for an additional two days. We used this 8-day

117 post-infection time point as our latent time point. Latent cultures had equivalent levels of

118 viral DNA copy numbers relative to host DNA (Figure 2A), suggesting that the number

119 of viral genomes capable of establishing latency remains similar despite oxygen

120 concentration, at least in this model system where ACV is used to promote latency

121 establishment. Unlike most viral transcripts, one viral long noncoding RNA, known as

122 the latency-associated transcript (LAT), is abundantly expressed during latency and can

123 therefore be used to measure HSV latent infection (7). In parallel with latent viral DNA

6

124   load, LAT expression was not statistically significantly different between the two

125   conditions (Figure 2B). Therefore, culturing primary neurons did not impact the ability of

126   HSV to establish a latent infection.

127

128   Following latency establishment and analysis, reactivation was induced by PI3-

129   kinase inhibition using LY294002, a well-characterized trigger of HSV-1 reactivation

130   which mimics loss of a branch of the nerve growth factor (NGF) signaling pathway in

131   neurons (8). HSV-1 reactivation proceeds in a two-step process. Phase I gene

132   expression precedes "full reactivation" (or "Phase II") as a transcriptional burst of all

133   classes of lytic viral genes (1, 9-12). Full reactivation is characterized by an ordered

134   transcriptional cascade wherein viral immediate early gene transcription precedes and

135   is essential to early gene expression, which is required for viral DNA replication, and

136   subsequent late gene transcription. Full reactivation is characterized by viral DNA

137   replication, viral protein synthesis, and ultimately new infectious virus production.

138

139   Phase I was interrogated through lytic gene expression analysis at 18 hours

140   post-treatment. Phase I gene expression was similar between the two conditions

141   (Figure 2D-F), with a statistically nonsignificant trend towards lower viral transcription

142   under physioxic conditions. Stayput-GFP has both a glycoprotein H (gH) deletion, which

143   renders the virus deficient in cell-to-cell spread and a GFP tag on late viral protein US11

144   (1). Therefore, Stayput-GFP permits the quantification of individual fully reactivating

145   neurons. The number of Us11-GFP-positive neurons was quantified at 48 hours post-

146   treatment with LY294002, which is indicative of Phase II reactivation. Interestingly, full

7

147   reactivation was significantly reduced in physioxic versus atmospheric cultures (Figure

148   2C). The average number of reactivating neurons was approximately 82 per well under

149   atmospheric conditions versus 12 in physioxic conditions, demonstrating a 7-fold

150   decrease under physioxia. Therefore, culturing neurons in more physioxic conditions

151   results in equivalent latency and Phase I reactivation but reduced progression to full

152   Phase II reactivation.

153

154   In our hands, the culturing of neurons under more physioxic conditions was

155   necessary to improve neuronal health for longer-term latency studies. Physioxic

156   conditions will also permit more consistent survival in microfluidic chambers, allowing

157   long-term studies using axonal-specific treatments or infections. This report highlights

158   the importance of accounting for oxygen concentration while studying the biology of

159   primary neurons and microbial infections in all cell types.

160

161

162   Materials and Methods

163

164   **Primary neuronal cultures**

165   Sympathetic neurons from the Superior Cervical Ganglia (SCG) of post-natal day 0-2

166   (P0-P2) CD1 Mice (Charles River Laboratories) were dissected as previously described

167   (13). Rodent handling and husbandry were carried out under animal protocols approved

168   by the Animal Care and Use Committee of the University of Virginia (UVA). Ganglia

169   were briefly kept in Leibovitz's L-15 media with 2.05 mM l-glutamine before dissociation

170   in collagenase type IV (1 mg/ml) followed by trypsin (2.5 mg/ml) for 20 min; each

171   dissociation step was at 37°C. Dissociated ganglia were triturated, and approximately

172   10,000 neurons per well were plated onto rat tail collagen in a 24-well plate.

173   Sympathetic neurons were maintained in feeding media: Neurobasal® Medium

174   supplemented with PRIME-XV IS21 Neuronal Supplement (Irvine Scientific), 50 ng/ml

175   Mouse NGF 2.5S (Alomone labs), 2 mM l-Glutamine, and 100 µg/ml Primocin

176   (Invivogen). Aphidicolin (3.3 µg/ml) was added to the media for the first five days post-

177   dissection to select against proliferating cells. In select experiments, neurons were

178   plated in microfluidic chambers. Briefly, standard photolithography was used to fabricate

179   channel array devices as described previously (14). Sylgard 184 (Dow Corning) was

180   prepared according to the manufacturer's recommended procedure and poured into the

181   mold. After curing at 95□°C for at least 72 hours, individual polydimethylsiloxane

182   (PDMS) chambers were cut out, sterilized in 70% EtOH and placed upon glass

183   coverslips coated with poly-D-lysine (100 µg/mL) and laminin (2 µg/mL).

184

9

Preparation of HSV-1 virus stocks

Stocks of Stayput Us11-GFP (strain SC16) for *in vitro* experiments were propagated

and titrated on gH-complementing F6 cells (5). Vero F6 cells were maintained in

Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FetalPlex

(Gemini Bio-Products) and 250 µg/mL of G418/Geneticin (Gibco).

Establishment and reactivation of latent HSV-1 infection in primary neurons

P6-8 SCG neurons were infected with Stayput Us11-GFP at MOI 10 PFU/cell,

(assuming 10,000 cells per well) in Dulbecco's Phosphate Buffered Saline (DPBS) +

$CaCl_2$ + $MgCl_2$ supplemented with 1% fetal bovine serum, 4.5 g/L glucose, and 10 µM

acyclovir (ACV) for 3.5 h at 37°C. The inoculum was replaced with feeding media (as

described above) with 50 µM ACV. 6 days post-infection, ACV was washed out and

replaced with feeding media alone. Reactivation was reported by quantifying the

numbers of GFP-positive neurons following the addition of 20 µM LY294002 (Tocris) or

60 µM forskolin (Tocris).

Analysis of viral DNA load and mRNA expression by reverse transcription–
quantitative PCR (RT–qPCR)

To assess the relative expression of HSV-1 mRNA, total RNA was extracted from

approximately 10,000 neurons using the Quick-RNA™ Miniprep Kit (Zymo Research)

with an on-column DNase I digestion. mRNA was converted to cDNA using the Maxima

First Strand cDNA Synthesis Kit for RT-qPCR (Fisher Scientific), using random

hexamers for first-strand synthesis and equal amounts of RNA (20–30 ng/reaction). To

10

208   assess viral DNA load, total DNA was extracted from approximately 10,000 neurons

209   using the Quick-DNA™ Miniprep Plus Kit (Zymo Research). qPCR was carried out

210   using PowerUp™ SYBR™ Green Master Mix (ThermoFish Scientific). The relative

211   mRNA or DNA copy number was determined using the comparative CT ($\Delta\Delta$CT) method

212   normalized to mRNA or DNA levels in latently infected samples. Viral RNAs were

213   normalized to mouse reference gene mGAPDH RNA. All samples were run in triplicate

214   on an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System with the

215   mean fold change compared to the calculated reference gene. The sequence of both

216   forward and reverse primers used have been published previously (5).

217

218   **Immunofluorescence**

219   Neurons were fixed for 15 min in 4% formaldehyde and blocked for 1 hour in 5%

220   bovine serum albumin and 0.3% Triton X-100, and incubated overnight in primary

221   antibody (Beta III Tubulin, EMD Millipore Cat #9354 at 1:500). Following primary

222   antibody treatment, neurons were incubated for 1 h in Alexa Fluor 488-conjugated

223   secondary antibodies for multicolor imaging (Invitrogen). Nuclei were stained with

224   Hoechst 33258 (Life Technologies). Images were acquired using an sCMOS charge-

225   coupled device camera (pco.edge) mounted on a Nikon Eclipse Ti inverted

226   epifluorescent microscope using NIS-Elements software (Nikon). Images were analyzed

227   using ImageJ.

228

229   **Statistical Analysis**

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230   Power analysis was used to determine the appropriate sample sizes for statistical

231   analysis. All statistical analysis was performed using Prism V10. Normality of the data

232   was determined with the Kolmogorov-Smirnov test. Specific analyses are included in

233   the figure legends.

234

235   **Acknowledgments**

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285

286   Figure legends

287   **Figure 1**

288   Neuronal Health in Atmospheric versus Physioxic Incubation Conditions.

289   (A-E) Primary sympathetic neurons were incubated in atmospheric (approximately 18%

290   oxygen) or physioxic (5% oxygen) conditions. (A) Neurons were latently infected with

291   Stayput-GFP at an MOI of 10 PFU/cell in the presence of acyclovir (ACV; 50 µM) for ten

292   days and cultures were imaged using phase contrast to document soma morphology.

293   (B-C) Neurons were incubated for up to 30 days post-plating. Scores representing cell

294   body (B) and axon (C) health were recorded over time. Biological replicates from 2

295   independent dissections; statistical comparisons were made using 2-way ANOVA. (D-E)

296   Neurons were plated in microfluidic chambers. 10 days post-plating, cultures were fixed

297   and stained for neuronal marker Beta III tubulin (white) (D). Scale bar 100 µm. Axonal

298   degeneration was analyzed using these images (E). Biological replicates from 4

299   independent experiments; statistical comparisons were made using unpaired non-

300   normal t-test. Individual biological replicates along with the means and SEMs are

301   represented. * P $<$ 0.05; ** P $<$ 0.01.

302

303   **Figure 2**

304   Physioxic incubation conditions reduce HSV-1 reactivation.

305   (A-F) Primary neurons were latently infected with Stayput-GFP at an MOI of 10 PFU/cell

306   in the presence of acyclovir (ACV; 50 µM) for six days and then reactivated two days

307   after the removal of acyclovir with LY294002 (20 µM). Quantification of relative latent

308   viral DNA load (A) and LAT expression (B) at 8 days post-infection. C) Quantification of

309   the number of GFP-positive neurons at 48 hours post-stimulus. D-F) Relative viral gene

310   expression at 18 hours post-stimulus compared to latent samples quantified by RT-

311   qPCR for immediate early viral gene ICP27 (D), early viral gene ICP8 (E), or late gene

312   gC (F) normalized to cellular control mGAPDH. Biological replicates from 4 independent

313   dissections; statistical comparisons were made using paired t-tests. Individual biological

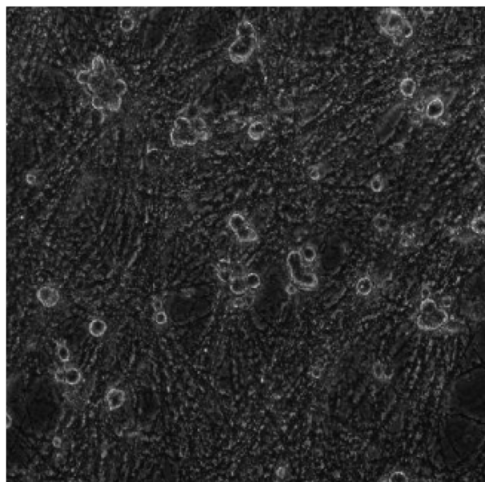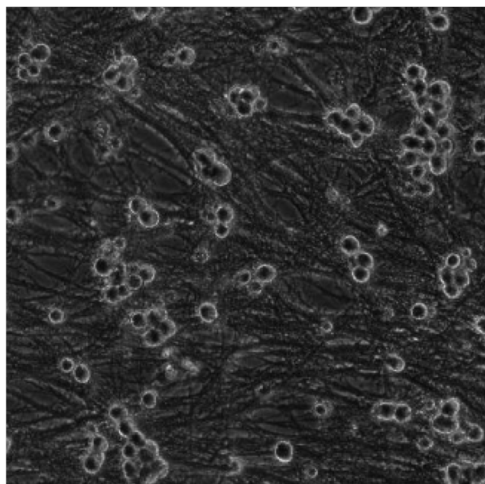314   replicates along with the means and SEMs are represented. * P□<□0.05; ** P□<□0.01.
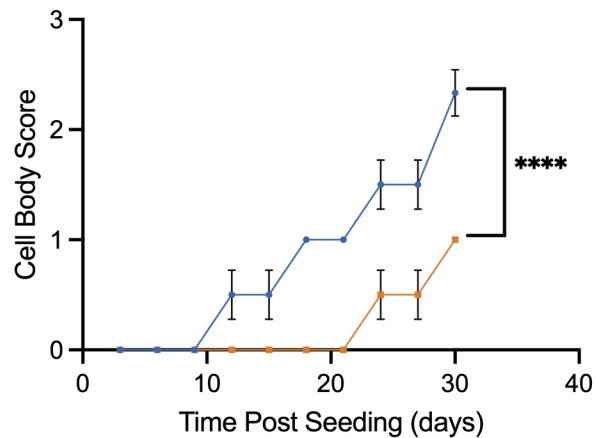
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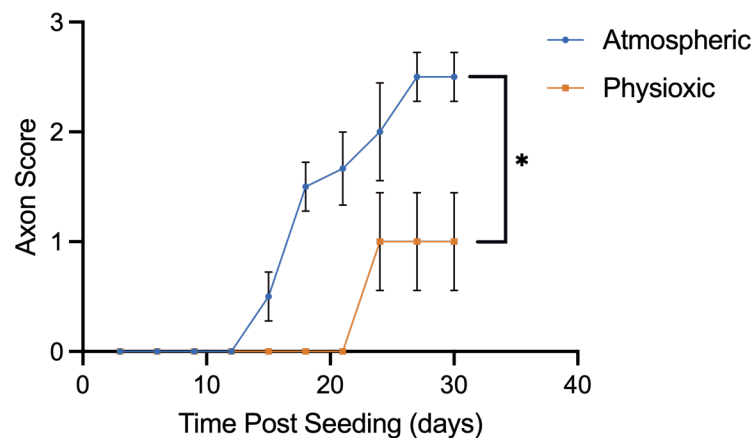316

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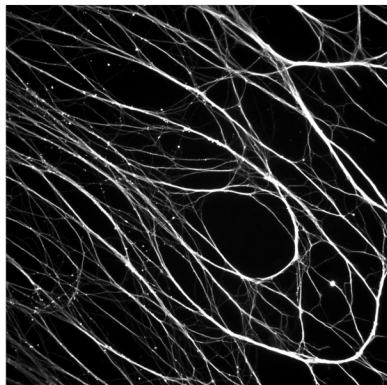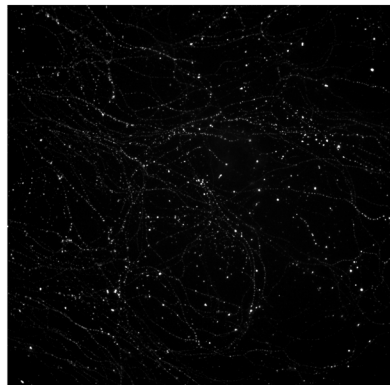
A. Atmospheric / Physioxic

B. Cell Body Health Over Time

C. Axonal Health Over Time

D. Atmospheric / Physioxic

E. Axonal Degeneration

A. Latent Viral Genomes

0.4830

Relative Copy No. vs Oxygen Conditions

B. Latent Viral RNA

Expression Normoxia/Atmospheric vs LAT, ICP27, ICP8, gC

C. Phase II Reactivation (48 hrs)

****

Number of GFP+ neurons vs Oxygen Conditions

Atmospheric
Physioxic

D. Phase I Reactivation *ICP27* mRNA (18 hrs)

0.8750

Relative Expression vs Oxygen Conditions

E. Phase I Reactivation *ICP8* mRNA (18 hrs)

>0.9999

Relative Expression vs Oxygen Conditions

F. Phase I Reactivation *gC* mRNA (18 hrs)

0.2500

Relative Expression vs Oxygen Conditions

Atmospheric
Physioxic