HSV-1 Helper Virus 5dl1.2 Suppresses Sodium Currents in Amplicon-Transduced Neurons

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White, Benjamin H., Theodore R. Cummins, Daniel H. Wolf, Stephen G. Waxman, David S. Russell, and Leonard K. Kaczmarek. HSV-1 helper virus 5dl1.2 suppresses sodium currents in amplicon-transduced neurons. J Neurophysiol 87: 2149–2157, 2002; 10.1152/jn.00498.2001. The Herpes Simplex Virus-1 (HSV-1) amplicon system is one of several viral-based strategies currently being developed for gene delivery into mammalian neurons for experimental or therapeutic purposes. Amplicon-containing viruses contain no HSV-1 genes and are amplified in titer relative to the helper viruses used to package them. In this way, they are designed to have a minimal impact on the physiology of transduced neurons. We show here, however, that amplicon preparations made using the 5dl1.2 helper virus selectively suppress sodium currents in cultured neurons by approximately 80% within 2 days of transduction and reduce average spike frequency in response to depolarization from 23 ± 4 to 0.4 ± 0.4 Hz. We observe similar suppression of Na⁺ currents in cells treated with the 5dl1.2 helper virus alone, indicating that the helper virus retains the ability of wild-type HSV-1 to inhibit these currents potently. Staining amplicon-transduced neurons with anti-HSV antibodies, we find that 80% of the neurons express viral proteins, indicating that helper virus typically co-infects these cells. We conclude that Na⁺ current suppression by the amplicon preparation results from the preferential coinfection of transduced neurons by the 5dl1.2 helper virus.

INTRODUCTION

Herpes Simplex Virus-1 (HSV-1) is a neurotropic virus that normally infects dorsal root ganglion cells (DRGs) in host animals but can infect a broad range of other neurons. Its broad selectivity, ability to infect postmitotic neurons, and capacity to accommodate large fragments of foreign DNA make it an attractive vehicle for the delivery of foreign genes into neurons for therapeutic or experimental purposes (see Fink et al. 1996; Kaplitt and Makimura 1997). The cytotoxicity of the wild-type virus, however, requires that some, or all, HSV-1 genes be removed from vectors derived from it to allow their use for gene transfer. Minimal vectors, which package bacterial plasmids, or “amplicons,” into an HSV-1 coat, represent a particularly elegant solution to this problem (reviewed by Ho 1994).

HSV-1 amplicons are plasmids that have been genetically modified to include viral control elements but that completely lack HSV-1 genes. Amplicons contain signals for DNA replication and viral packaging and an HSV-1 immediate early gene promoter to drive expression of any gene(s) of interest inserted downstream. HSV-1 amplicons are replicated and packaged into viral capsids in permissive cell lines with the aid of replication-defective HSV-1 helper viruses. In addition to amplicon-containing virus particles, this process also produces more helper virus, which remains as part of the final preparation. Procedures for making amplicon preparations free of helper virus have been developed but lower yields have limited their utility (see Robbins and Ghivizzani 1998; but also Wang et al. 2000).

Although helper viruses are a common component of amplicon preparations, little has been reported on the frequency with which they co-infect amplicon-transduced neurons under typical experimental conditions or on their cytopathological effects on co-infected neurons. While it is clear that the cytotoxicity of helper viruses is significantly attenuated relative to that of wild-type HSV-1 (Lim et al. 1996), sublethal effects on cellular physiology have also not generally been investigated. The successful use of amplicon preparations to express, or overexpress, genes of interest in neurons both in vitro and in vivo (Meier et al. 1997; Neve et al. 1997; Phillips et al. 1999; Song et al. 1998) has no doubt blunted interest in these issues, particularly as the effects of helper virus can be controlled for by amplicon preparations lacking the gene of interest. The results we report here suggest, however, that the physiological effects of helper virus on amplicon-transduced neurons call for increased caution in matching the titers of helper virus in control and experimental preparations to ensure that results are comparable.

It has long been known that HSV-1 infection results in the rapid loss of neuronal excitability. Early evidence suggesting that this loss resulted from the downregulation of voltage-sensitive Na⁺ currents (Fukuda and Kurata 1981; Mayer 1986; Oakes et al. 1981) has been confirmed by more recent work (Howard et al. 1998). In the course of using amplicons to overexpress K⁺ channels in cultured rat cortical neurons, we observed widespread suppression of inward current in amplicon-transduced neurons, coupled with an almost complete loss of excitability. Surprisingly, we obtained the same result with an amplicon preparation expressing only Green Fluorescent...
Protein (GFP), suggesting that the effect was not due to increased K⁺ conductance. Because the suppression of excitabil-
ity by wild-type HSV-1 is known to depend on viral gene expres-
sion, infection by amplicon-containing virus alone is not ex-
etected to exert this effect. Instead, we have traced the
suppression to the 5dl1.2 helper virus used in amplicon pack-
aging. We demonstrate that 5dl1.2, like wild-type HSV-1,
inhibits voltage-sensitive Na⁺ currents in infected neurons.
Consistent with our physiological findings, we directly de-
 monstrate a high-frequency of co-infection of amplicon-trans-
duced neurons by helper virus, even at low multiplicities of
infection (MOI = 0.2). Using anti-HSV antibodies to detect
helper-virus-infected neurons treated with the GFP-expressing
amplicon preparation, we find that helper virus co-infects 80% of
amplicon-transduced cortical neurons under our standard
conditions. Because the loss of electrical activity in amplicon-
transduced neurons may under some circumstances be mis-
taken for the effects of an introduced gene, our results imply
that 5dl1.2 titers in experimental and control amplicon prepara-
tions should be matched to produce equal degrees of co-
infection. In addition, we describe an immunofluorescence
assay that should prove more useful than the standard plaque
 assay in determining accurate titers of helper virus on the
neurons of interest.

METH ODS

Cell culture

Whole cerebral cortices from newborn (P0) Sprague Dawley rats
were dissected free of meninges, washed in Hank’s balanced salt
solution on ice, then digested with 0.05% trypsin/0.53 mM EDTA at
37°C for 20 min. (Unless otherwise noted all reagents here and in the
following text were from Gibco, Grand Island, NY.) The cell suspen-
sion was then diluted 1:1 into Dulbecco’s modified Eagle’s medium
(DMEM) and treated briefly with DNase I to disperse tissue clumps
prior to trituration with a fire-polished Pasteur pipette and passage
through a Falcon cell strainer (Applied Scientific, South San Fran-
cisco, CA). Dissociated cells were pelleted by centrifugation at 1,000
g for 5 min and resuspended in DMEM/5% bovine serum (FBS)
after removal of the supernatant. This procedure was repeated twice,
and the cell pellet was finally resuspended in Neurobasal medium
supplemented with B27, 5% FBS, 2 mM l-glutamine, 1 mM sodium
pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml). The
cell suspension was plated at a density of 100,000 cells/well in
24-well plates containing polyornithine (0.01 mg/ml)- and laminin
(2 μg/ml)-coated glass coverslips. Long-term cultures were fed every
day by exchanging half the media with fresh media.

Adult rat dorsal root ganglion (DRG) neurons were cultured as
previously described (Caffrey et al. 1992). Briefly, the L₄ and L₅ DRG
ganglia were harvested from adult male Sprague-Dawley rats. The
DRG were treated with collagenase (1 mg/ml) for 25 min and colla-
genase (1 mg/ml) plus papain (30 U/ml) for 25 min, dissociated in
DMEM and Ham’s F₁₂ medium supplemented with 10% FBS and
plated on glass coverslips. The cultures were maintained at 37°C in a
humidified 95% air-5% CO₂ incubator.

Virus preparation and titering

Amplicon preparations were prepared as described by Lim and
Neve (1999). Briefly, amplicon DNA containing the gene for Green
Fluorescent Protein (GFP) was transfected into cultured 2-2 Vero cells
using 2 μg maxiprep DNA and 12 μl lipofectamine in 1.0 ml of
serum-free media; serum containing media was added after 5 h. Prior
to transfection, 2-2 cells were grown in 30-mm culture dishes until
80% confluent, at 10% CO₂, in DMEM media (Hyclone Laboratories,
Logan, UT) containing 10% fetal calf serum (FCS), and 1% Pen/
Strep. Twenty hours after transfection, 20 μl (1 × 10⁶ pfu) of helper
virus was added, and 36 h later cells were lysed, and this lysate was
added onto fresh 2-2 cells. This amplification process was repeated
three times, resulting in a final 60 ml of virus-containing cell lysate.
The lysate was purified over a sucrose step gradient (10%/30%/60%)
by centrifugation at 125,000 g for 1 h. The virus bands at the 30%/60%
interface were removed and diluted 1:10 into PBS. Virus was then
concentrated by ultracentrifugation at 125,000 g for 1 h. The resulting
pellet was resuspended in 200 μl PBS/10% sucrose and stored at
−80°C in 30-μl aliquots. Unpurified helper virus preparations were
made by infecting untransfected 2-2 cells with 5dl1.2 helper virus as
described in the preceding text and isolating the virus-containing
lysate. Control lysates were prepared from uninfected 2-2 cells.

Plaque assays to determine the titer of 5dl1.2 helper virus in the
viral preparations were carried out as follows. A 100 μl aliquot of
viral preparation was added to a 6-cm dish containing 1 × 10⁶ 2-2
cells in 2 ml DMEM/10% FCS. After allowing the virus to adsorb for
2 h, the media was replaced with 3 ml 1% Seaplaque agarose (Bio-
Whittaker Molecular Applications, Rockland, ME) at 40°C in
DMEM/5% FCS. Agarose was allowed to solidify before returning
the culture dishes to a 37°C incubator for 24 h. DMEM/5% FCS (2
ml) was added after 24 h and exchanged again after a further 24 h, and
1 day later cells were fixed with 3 ml 5% methanol/10% acetic acid
for 30 min. Plaques were visualized by staining with crystal violet and
counted. Amplicon titers were determined by infecting PC12 cells,
plated in the wells of a 24-well culture dish at a density of 500,000
cells/well, with up to 4 μl of HSV-GFP for 24 h. One day after
infection, cells were fixed with 4% paraformaldehyde/0.1 M PBS for
25 min then washed with PBS. Amplicon-transduced cells were
identified by the presence of GFP fluorescence using an Olympus
BX-60 fluorescence microscope and counted.

Viral transduction/infection

TRANSUCTION. Gradient-purified GFP-HSV amplicon prepara-
tions were stored at −80°C and thawed at 37°C before dilution into
media and application to neuronal cultures in a volume of 300 μl. The
estimated ratio of virus particles per neuron, or MOI, used for trans-
duction of the cortical neuron cultures was 0.2 but was higher for
DRG neurons, which were plated at lower (and variable) densities.
Neuronal cultures were incubated for 6 h with the amplicon prepara-
tion prior to washout, and electrophysiological recordings were car-
rried out 2 days after transduction except where otherwise noted.
Cortical neurons were typically transduced 6–7 days after plating.

Cortical neuron cultures were infected with helper virus according
to a similar protocol. Cultures were treated with a volume of 2-2 cell
lysate, from cells infected with 5dl1.2 helper virus, sufficient to give
a multiplicity of infection of 2.3 (a condition determined by immu-
nostaining with anti-HSV antibodies to yield infection of 67% of the
cultured cells). Control cultures received an equal volume of 2-2
lysate from cells that had not been infected with 5dl1.2 helper virus.
In experiments to determine the pattern of immunostaining of anti-
HSV antibodies, which were carried out in the presence or absence of
10 μg/ml cycloheximide to block the synthesis of viral proteins,
neuronal cultures were preincubated with CHX for 1 h followed by a
5 h incubation with helper virus.

Whole cell patch-clamp recording of neurons

CORTICAL NEURONS. Whole cell patch-clamp recordings were con-
ducted at room temperature using an Axopatch 1D amplifier and
pCLAMP 6 data-acquisition software (both from Axon Instruments,
Foster City, CA). Patch electrodes were pulled using a vertical Na-
rishige glass microelectrode puller and had resistances of 3–4 MΩ.
The offset potential was zeroed before patching the cells. Voltage errors were minimized using 80–90% series resistance compensation. The capacitance artifact after breakthrough was cancelled by capacitance compensation and the magnitude of compensation applied was used to estimate cell membrane capacitances. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. The pipette solution contained (in mM) 97.5 K gluconate, 32.5 KCl, 5 EGTA, and 10 HEPES, pH 7.2, and the bath solution contained (in mM) 140 NaCl, 1.3 CaCl<sub>2</sub>, 5.4 KCl, 25 HEPES, and 33 glucose, pH 7.2. Voltage-clamp data were sampled at a rate of 8 kHz and filtered at 2 kHz. Amplicon-expressing neurons, identified as GFP-positive by fluorescence microscopy, were recorded 2 days after transduction except where noted, and control cells were handled in parallel but were not incubated with the amplicon preparation. To identify the inward currents, voltage-clamp recordings were made in 10 control cells before and after bath application of 250 nM tetrodotoxin (TTX). Neurons from 5dl1.2-helper-virus-infected cultures were picked at random and recorded 1 day after helper-virus treatment. This was prior to the onset of extensive neurite degeneration and cell death, which was evident by 2 days. Neurons from parallel cultures treated with a cell lysate from 2-2 helper cells were uninfected with 5dl1.2 helper virus were used as controls.

**RESULTS**

**Suppression of neuronal Na<sup>+</sup> currents by an amplicon preparation**

To investigate the physiological effects of HSV-1 amplicon preparations on neurons, we treated cultured rat cortical neurons with an amplicon preparation containing the gene for GFP and recorded from amplicon-transduced neurons identified by the presence of green fluorescence (Fig. 1A). Initial current-clamp recordings from neurons 2 days after transduction revealed a significant difference in the excitability of the amplicon-transduced neurons compared with that of controls (Fig. 1, A). Initial current-clamp recordings from neurons 2 days after transduction revealed a significant difference in the excitability of the amplicon-transduced neurons compared with that of controls (Fig. 1, B).
Suppression of Na\(^+\) currents by wild-type HSV-1 is well established in adult DRG neurons (Howard et al. 1998; Mayer et al. 1986), the natural host cell of this virus, and although live virus was absent from our amplicon preparation, the effect we observed with cortical neurons appeared similar. To investigate whether the amplicon preparation, like live HSV-1, suppressed Na\(^+\) currents in DRG neurons, we transduced cultured DRG neurons from adult rat and 2 days later conducted whole cell recordings under conditions designed to isolate Na\(^+\) currents. Consistent with the results obtained in cortical neurons, we observed a 78% inhibition of the Na\(^+\) current in transduced cells versus untransduced controls (Fig. 3, A and B).

To determine whether transduced neurons recovered their inward currents over time, we compared the normalized Na\(^+\) currents in transduced cortical neurons 1–2 wk posttransduction with those observed 1–2 days posttransduction. As indicated in Fig. 4, there is, at later times (Fig. 4C, B and C). Whereas 90% of control cells fired multiple action potentials in response to depolarizing current injections of \(\leq 300\) nA (average peak firing frequency: 23 ± 4 Hz), 88% of the transduced neurons were completely unexcitable and the remaining 12% fired a single spike. Examination of whole cell currents in the amplicon-transduced neurons strongly suggested that the suppression of excitability derived from a profound inhibition of the inward currents (Fig. 2A). Peak inward currents in amplicon-transduced neurons declined sevenfold relative to those of uninfected control cells. In contrast, steady-state outward currents declined less than twofold, a difference that was not statistically significant (Fig. 2C). We identified the inward currents in these cells as Na\(^+\) currents, using the specific Na\(^+\) channel blocker tetrodotoxin (TTX; Fig. 2B). Application of 250 nM TTX to control cells selectively inhibited 97% of the inward current \((I_{\text{inward}} = 5.7 ± 0.8\) nA before TTX vs. 0.13 ± 0.05 nA after TTX; \(n = 10\)).

**FIG. 2.** The loss of excitability on HSV amplicon transduction results from suppression of Na\(^+\) currents in embryonic rat cortical neurons. A: representative whole cell currents from voltage-clamped cortical neurons. Shown are currents from control (left) and amplicon-treated (right) cells from parallel cultures. Cells were held at −70 mV and stepped to +80 mV in 10-mV increments. B: currents from a control cell measured before (left) and after (right) application of 250 nM TTX indicate that the inward currents inhibited by the amplicon preparation are Na\(^+\) currents. Scale bar same as in A. C: peak Na\(^+\) (left) and outward (right) currents were normalized to cell capacitance and averaged for control and amplicon-transduced cells. (For Na\(^+\) currents, \(P < 10^{-4}\) by 2-tailed \(t\)-test.)

**FIG. 3.** Na\(^+\) currents are suppressed in amplicon-transduced dorsal root ganglion cells (DRGs). A: representative Na\(^+\) currents from amplicon-transduced (bottom) and parallel control (top) DRGs. L\(_1\) and L\(_2\) lumbar DRGs were cultured from adult rats and treated with amplicon as described in METHODS. Na\(^+\) currents were recorded by whole cell voltage clamp from small-diameter neurons (18–25 µm) in the presence of 140 mM CsCl and 100 µM cadmium to block K\(^+\) and Ca\(^{2+}\) currents, respectively. Cells were held at −100 mV and stepped from −80 mV to +40 mV in 10-mV increments. B: average Na\(^+\) currents from control (\(n = 22\)) and amplicon-transduced (\(n = 22\)) cells (\(P < 5 \times 10^{-3}\) by 2-tailed \(t\)-test).
A profound reduction in the percentage of neurons with small current densities (normalized values of 0.0–0.4) when compared with neurons assayed 1–2 days posttransduction (43 vs. 70%, Fig. 4C, top). As is also indicated in Fig. 4, the loss of transduced neurons with small inward current densities is paralleled by a rapid decline in the number of GFP-labeled neurons (Fig. 4A). We found that within 4 days of transduction the number of green neurons declined by more than 50%. By 1 wk after transduction, less than 20% of the original number of labeled neurons remained. Some of the loss of label is likely due to downregulation of GFP expression in transduced neurons, a common feature of amplicon-expressed genes (Lim et al. 1996), but morphological characteristics of many of the labeled neurons suggested that they were dying as many of them showed signs of rounding and neurite degeneration (Fig. 4B). The electrophysiological results further suggest that the amplicon-transduced neurons fall into two classes: a class with very low Na+/H+ current densities that degenerate and perhaps die, and another more viable population with Na+/H+ current densities that are less severely depressed.

**Immunodetection of helper-virus infection in amplicon-treated neurons**

Although wild-type virus was absent from our preparation, the 5dl1.2 helper virus used to prepare it was present at a titer similar to that of amplicon-containing virus as determined by the standard plaque and fluorescence assays described in METHODS (1.2 × 10^8 pfu/ml vs. 1.0 × 10^8 infectious units ifu/ml, respectively). While ratios of amplicon-containing virus to helper virus of 10:1 have been reported in enriched amplicon preparations (Kwong and Frenkel 1995), 1:1 ratios are typical (Ho 1994; Smith et al. 1995). Because the 5dl1.2 helper virus has previously been reported to reduce neuronal viability with a similar time course to our observed loss of GFP fluorescence and because it expresses viral genes, which is known to be required for the suppression of Na+ currents (Mayer et al. 1986), it seemed possible that both the observed reductions in viability and excitability derived from co-infection of the transduced neurons with 5dl1.2 helper virus. To test this possibility, we developed an immunofluorescence technique using anti-HSV antibodies to simultaneously measure infection by both amplicon and helper virus.
Because infection of neurons by helper virus results in the expression of viral genes, whereas infection by amplicon-containing virus does not, cells infected with helper virus should be selectively immunoreactive to antibodies against HSV secondary proteins. To test the ability of anti-HSV antibodies to detect helper virus infection and to distinguish it from virion attachment, which will also occur with amplicon-containing virus, we incubated cortical neuron cultures with 5dl1.2 helper virus for 5 h in the presence or absence of cycloheximide (CHX, at 10 μg/ml) to block viral protein synthesis. Cells were then fixed and immunolabeled with anti-HSV antibodies and a fluorescein-labeled secondary antibody. In the absence of CHX, 9% of the cells showed clear anti-HSV immunofluorescence with the cell bodies typically filled and neurites more lightly labeled (Fig. 5A). In contrast, none of cells in the CHX-treated cultures showed this pattern of staining (Fig. 5B), although some were lightly “decorated” with dots, which may represent staining of the structural proteins of attached virions. Both patterns of staining were enhanced by extending the incubation with helper virus to 24 h (Fig. 5, C and D), a condition that led to the labeling of 37% of cells in the absence of CHX. A small number of cells (0.8%) in the 24-h CHX-treated cultures showed dim, somatic immunofluorescence, but this labeling was easily distinguishable from that seen in cells untreated with CHX and is likely to be due to residual protein synthesis in the prolonged presence of helper virus. Confident that we could discriminate between infected cells and those having only attached viral particles, we used the anti-HSV antibodies to selectively identify helper virus-infected neurons in our amplicon-treated cultures.

To examine the degree of helper-virus infection of neurons treated with our amplicon preparation, we incubated cortical neuron cultures at MOIs (0.2) previously found to suppress Na+ currents in approximately 70% of transduced cells (Fig. 4C, top). Incubation with amplicon was carried out for 6 h prior to 24 h washout, and neurons were allowed to grow for a further 48 h prior to examination by immunofluorescence. Amplicon-transduced neurons were visualized by green fluorescence (Fig. 6, A and D). As described in the preceding text, helper-virus-infected neurons were identified by immunofluorescence using anti-HSV antibodies but this time with a Texas-red-labeled secondary antibody (Fig. 6, B and E). Coincident labeling of cells could readily be evaluated by overlapping the signals in the two fluorescence channels with double-labeled neurons appearing yellow (Fig. 6, C and F).

While only 3.3% of the neurons had been transduced by amplicon, we found that 80% of these GFP-positive neurons were also anti-HSV immunopositive (Fig. 6C). This was nearly an order of magnitude higher than the co-infection rate predicted from the helper-virus infection rate (10.2%) if all infections were to occur randomly and independently. Consistent with our physiological data, these results strongly indicate that viral particles containing amplicon and helper virus DNA preferentially co-infect the same neurons, even at the low MOI we have employed.

Interestingly, the relative titer of helper virus to amplicon-containing virus, when measured by the rates of infection of cortical neurons, was approximately threefold higher than that estimated from the titers derived from the standard assays. The rate of cortical neuron infection by helper virus (10.2%) corresponds to a titer almost sixfold more than that estimated by “plaque assay” on 2-2 Vero cells (6 × 10^3 ifu/ml vs. 1.2 × 10^8 pfu/ml), while the rate of amplicon transduction (3.3%), corresponds to an infectious titer of 2 × 10^8 ifu/ml, or twice that measured by GFP fluorescence on amplicon-treated PC12 cells (1.0 × 10^6 ifu/ml). This suggests that the standard assays may differentially underestimate helper virus titers, which will also lead to higher than expected rates of co-infection of amplicon-transduced neurons by helper virus. The discrepancy also highlights one of the advantages of the immunofluorescence assay employed here, namely, that it allows the titers of both amplicon-containing virus and helper virus to be determined on the same population of cells by similar techniques.

**Suppression of neuronal Na+ currents by helper virus alone**

The high frequency of co-infection of amplicon-transduced neurons by helper virus provides a rational explanation for the widespread suppression of Na+ currents in these cells if the 5dl1.2 helper virus, like wild-type HSV-1, downregulates these currents. To directly test the ability of 5dl1.2 to inhibit Na+ currents, we carried out patch-clamp recordings from cultured rat cortical neurons infected with helper virus alone. We incubated neuronal cultures with titers of 5dl1.2 helper virus suf-
ficient to infect 67% of the cells as determined by immunostaining with anti-HSV antibodies. Sampling inward currents in randomly selected cells by whole cell patch-clamp techniques 1 day later, we found that two-thirds of the cells recorded from (12/15) had Na⁺ current densities of less than or equal to −0.2 nA/pF (Fig. 7, A and B). Only one-fifth of control cells (3/13) had current densities this small, with the average Na⁺ current in the controls having a value of −0.53 nA/pF (Fig. 7, A and B). One-third of the cells treated with helper virus (3/15) had large inward currents (−0.93 nA/pF) and were presumably uninfected. The strong inhibition of Na⁺ currents in helper virus treated cells at a frequency similar to the frequency of infection, is strong evidence that 5dl1.2, like wild-type HSV-1, suppresses neuronal Na⁺ currents. We conclude that the 5dl1.2 helper virus is the cause of Na⁺ current suppression observed in amplicon-transduced neurons.

DISCUSSION

We report here several findings that, taken together, strongly support the conclusion that helper virus present in HSV-1 amplicon preparations can lead to the loss of Na⁺ currents and neuronal excitability. We find that within 2 days, 70% of the neurons transduced with an amplicon preparation packaged using the 5dl1.2 helper virus exhibit substantially suppressed voltage-sensitive Na⁺ currents. This effect is similar to the inhibition of Na⁺ currents reported for wild-type HSV-1 and is likely to derive from infection by either the amplicon-containing virions or the helper virus in the preparation. Downregulation of Na⁺ currents by native HSV-1 is known to require viral gene expression and is absent in strains in which specific genes are mutated or deleted (Howard et al. 1998; Mayer et al. 1986; Storey et al. 1996). The fact that the 5dl1.2 helper virus, but not the amplicon, carries viral genes therefore suggests that helper virus is responsible for the suppression of Na⁺ current in amplicon-infected neurons. This conclusion is confirmed by our observations that the helper virus has the capacity of the wild-type virus to suppress Na⁺ currents and that it broadly co-infects amplicon-transduced neurons under our experimental conditions.

We have not investigated the mechanism for the widespread co-infection of neurons by helper virus and amplicon-containing virus that occurs at an eightfold higher rate than would be predicted for random and independent infection events. Because amplicon DNA and helper virus DNA are thought to be packaged into independent, but otherwise identical, viral particles, however, the preferential co-infection of cells by amplicon-containing virus and helper virus must reflect the different susceptibilities of individual neurons to viral infection, perhaps due to differential expression of the viral receptor.

Differing susceptibilities to infection of different cell types may also underlie the conflicting estimates of viral titer we obtained using different assay types. The immunofluorescence technique introduced in this paper gave a helper virus titer sixfold higher on cortical neurons than that determined by the standard plaque assay performed on African Green Monkey Kidney cells (i.e., 2-2 Vero cells). Similarly, amplicon titers
determined directly on cortical neurons were twice those found by the standard assay using pheochromocytoma (PC12) cells. As a consequence the relative titer of amplicon to helper virus measured on cortical neurons was approximately three times that estimated by the usual methods. These observations highlight the advantages of the immunofluorescence technique for estimating viral titers introduced here. It not only permits titering the levels of contaminating helper virus directly on the cell type of interest but also allows the amplicon-containing virus to be titered on the same cell type by the same methodology as long as the expressed gene product is labeled (e.g., by GFP) or if antibodies to it are available. A more accurate estimate of the extent of helper virus contamination can then be obtained.

The mechanism of HSV suppression of Na\(^+\) currents is unknown. One possible mechanism involves the rapid internalization of Na\(^+\) channels, but using a variety of anti-Na\(^+\) channel antibodies we were not able to demonstrate any overt redistribution of immunoreactivity in amplicon-transduced neurons (data not shown). Na\(^+\) channel function, rather than surface expression, may thus be affected by HSV infection though further work will be necessary to address this possibility. Interestingly, not all channel types may be targets for suppression as we failed to observe suppression of Na\(^+\) currents by the amplicon preparation in a human embryonic kidney (HEK) cell line stably transfected with the muscle-type Na\(^+\) channel (data not shown). HEK cells may also lack regulatory factors required for mediating channel inhibition, but it remains an interesting possibility that only specific Na\(^+\) channel types are targeted by the virus. Investigation of this point may also provide clues as to which viral genes mediate Na\(^+\) current suppression.

Our data indicate that Na\(^+\) current suppression is a common feature of infection in neurons. Previous studies of Na\(^+\) current inhibition by wild-type HSV have typically focused on DRG neurons as these are the normal host for herpes simplex virus infections. Our observation that Na\(^+\) currents were similarly inhibited in both rat embryonic cortical neurons and adult DRG neurons indicates not only that the suppression of Na\(^+\) current occurs generally but that the co-infection of transduced neu-
rons by helper virus is also a general characteristic of treatment with the amplicon preparation. Also, we observed similar suppression of inward current with amplicon preparations containing transgenes other than GFP, demonstrating that the inhibition was not a property of the GFP-containing amplicon preparation reported on here. Indeed, we first observed the profound suppression of Na$^{+}$ currents during experiments to test the effects of expressing mutant K$^{+}$ channels on cortical neuron physiology (data not shown).

Overall, our results indicate that some care should be taken in interpreting the results of experiments using amplicon preparations because even substantial removal of contaminating helper virus and working at low MOIs do not guarantee elimination of effects of the helper virus. In particular, it seems critical that control preparations have titers of helper virus similar to those of the experimental amplicon preparation to ensure like frequencies of co-infection by helper virus. Otherwise, effects of co-infection seen with the experimental preparation may be erroneously attributed to the transgene. The immunofluorescence method introduced here to determine viral titers may also be helpful in establishing accurate relative titers of helper and amplicon-containing virus on the cell type to be infected.

What implications our results have for transduction experiments carried out in animals or for gene therapy is not yet clear. Indeed, amplicon preparations made with the 5dl1.2 helper virus have been successfully used in numerous gene transfer experiments in vivo without apparent deleterious consequences (Antonawich et al. 1999; Carlezon et al. 1997, 1998; Chen et al. 2001). Likewise, preliminary experiments on slice preparations from amplicon-expressing neurons in the locus coeruleus of rats have failed to show noticeable suppression of excitability in transduced neurons after 1 wk (G. Aghajanian, personal communication). Similarly, experiments involving the injection of an amplicon preparation into rat dentate gyrus have been reported to leave the population responses of both dentate gyrus and hippocampal neurons unchanged (Dumas et al. 1999). The helper virus used for these last experiments, however, differed genetically from 5dl1.2 and has not, to our knowledge, been tested for its ability to suppress Na$^{+}$ currents. It is, however, also possible that the loss of some transduced neurons due to co-infection can be tolerated in vivo or that neurons in vivo tolerate helper virus infection better than those in culture. Given the profound effects we observe in culture, however, possible effects of helper virus co-infection cannot necessarily be ignored.

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