



# Neuronal sprouting in mouse sensory ganglia infected with herpes simplex virus type 2 (HSV-2): induction of growth-associated protein (GAP-43) and ultrastructural evidence

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Herpes simplex virus (HSV) is neurotropic and when inoculated on the mouse footpad is retrogradely transported to the associated dorsal root ganglia (DRG), where infection is established. Previous observations suggest that, after HSV infection, sensory ganglion neurons may mount a sprouting response. In our HSV-infected DRG model, we investigate this issue by (1) examining expression of growth-associated protein (GAP-43), a molecule known to be induced by growing axons, and (2) determining ultrastructurally whether HSV-infected dorsal roots contain neurites. In a time course study, we show that GAP-43 is induced both in HSV-infected DRG and their central processes. The increase in GAP-43 is first seen 2 weeks following unilateral footpad inoculation in both cell bodies and dorsal roots, and is sustained at 1 month post inoculation in roots but not in perikarya. Large bundles of unmyelinated small caliber axons, lacking Schwann cell ensheathment, are observed by electron microscopy in dorsal roots 2 weeks and 1 month following inoculation. These profiles resemble developing or regenerating neurites and are rarely seen in roots of mock-infected or uninfected controls. The increased GAP-43 immunoreactivity and ultrastructural changes shown here, in conjunction with previously documented selective neuropeptide and enzyme alterations, confirm that a sprouting response is mounted in sensory ganglia following acute HSV infection.

**Keywords:** dorsal root ganglia; immunocytochemistry; Western blotting; electron microscopy

## Introduction

Herpes simplex viruses types 1 and 2 (HSV-1, -2) are neurotropic. Following peripheral inoculation, these viruses are retrogradely transported in nerve processes to the associated sensory ganglia, where acute and/or latent infection is established in neurons (Stevens and Cook, 1971; Cook *et al.*, 1974; McLennan and Darby, 1980; Stroop *et al.*, 1984).

Many neurobiological responses to HSV in sensory ganglia of infected animals have been documented during the acute and latent states in the weeks following inoculation. There is severe neuronal death (Henken and Martin, 1993) and an influx of inflammatory cells (Gebhardt and Hill, 1988, 1990). There are selective alterations of host neuropeptide

and enzyme constituents. Galanin is transiently increased (Henken and Martin, 1992a, b), calcitonin gene-related peptide (CGRP) is unaffected (Henken and Martin, 1992a), substance P (unpublished observation) and fluoride-resistant acid phosphatase (FRAP) are reversibly decreased (Tenser *et al.*, 1991). In a genital HSV infection model, neurite sprouting has been noted (Soffer and Martin, 1989). The selective neuropeptide and enzyme alterations and the presence of sprouts, are consistent with neurobiological responses seen during regeneration of peripheral processes following injury (Tenser, 1985; Hökfelt *et al.*, 1987, Villar *et al.*, 1989, 1991) suggesting that sensory ganglion neurons may enter a growth mode in response to neuronal loss induced by HSV infection.

To test this hypothesis directly, we have examined alterations in growth-associated protein (GAP-43) in mouse dorsal root ganglia (DRG) and dorsal

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roots, and examined these roots ultrastructurally, following unilateral HSV inoculation of the footpad. GAP-43 is a growth cone membrane protein (Skene *et al*, 1986) that is expressed at high levels in periods of axon growth during development and regeneration (Skene and Willard, 1981a, b; Jacobson *et al*, 1986; Verhaagen *et al*, 1986; Basi *et al*, 1987; Coggeshall *et al*, 1991; Gispén *et al*, 1991). We show that GAP-43 is induced in HSV infected ganglia and dorsal roots; moreover, the levels are similar to those seen in roots after sciatic nerve transection. In addition, we demonstrate that large numbers of unmyelinated neurites are present in dorsal roots following peripheral HSV inoculation. The induction of GAP-43 and the presence of sprouts are consistent with changes seen during regeneration in DRG following sciatic nerve axotomy (Coggeshall *et al*, 1991; Tanaka and Webster, 1991).

## Results

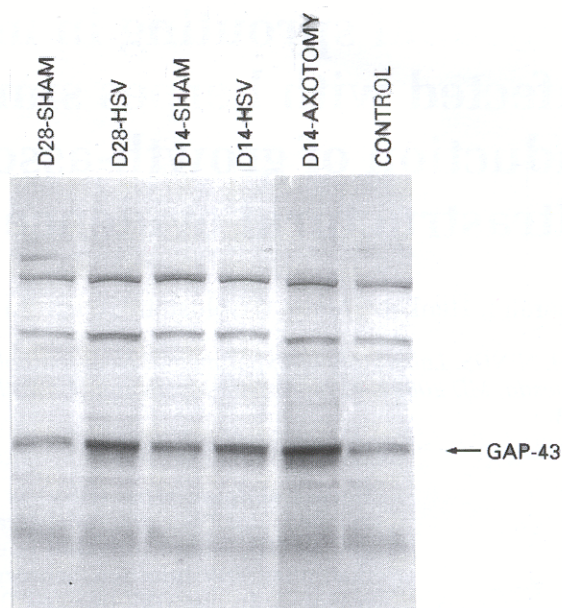
To compare relative differences in GAP-43 levels in HSV-2 infected ganglia and roots and their contralateral uninfected equivalents, a Western blot analysis was employed. Increased levels of GAP-43 were found in infected ganglia and roots by this method. To localize and quantify the increased levels of GAP-43, immunocytochemistry followed by image analysis was performed on decalcified longitudinal sections of spinal cord with their associated ganglia. Increased immunoreactivity was found in DRG perikarya as well as in dorsal roots, 2 weeks and 1 month following inoculation. Ultrastructurally, bundles containing very large numbers of unmyelinated fibers (> 100) were found in infected dorsal roots, but not in uninfected roots.

### Western blotting

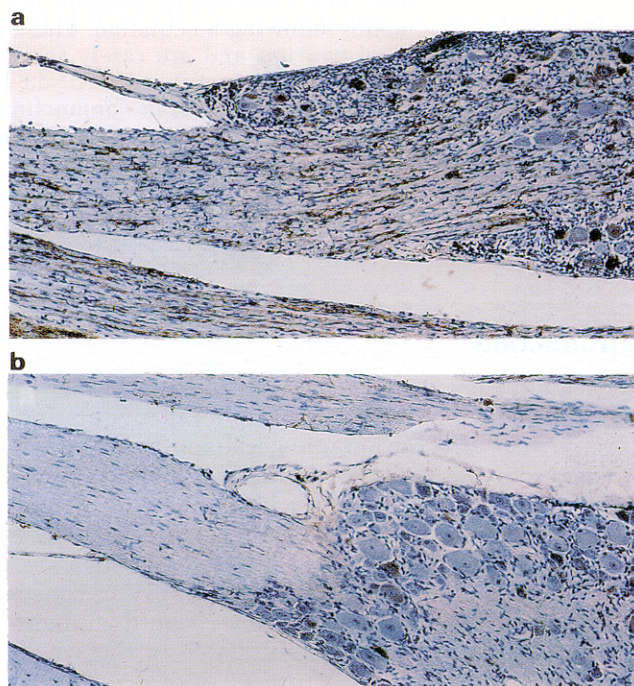
In an initial attempt to examine the relative differences in GAP-43 levels in HSV-2 infected and uninfected dorsal roots, a Western blot analysis was performed (Figure 1). Five days post inoculation (dpi) no difference is seen in the intensity of the bands (not shown). However, at 14 and 28 dpi a clear increase in the amount of GAP-43 immunoreactivity is seen when compared to either mock-infected or normal control mice. GAP-43 expression at 14 and 28 dpi was similar to that seen in dorsal roots 14 days after sciatic nerve transection. The specificity of the antiserum has been well documented (Curtis *et al*, 1991); however, the higher molecular weight immunoreactive bands, similar to proteins previously noted in peripheral nerve samples (Tetzlaff and Bisby, 1989; Curtis, unpublished observations), are currently unidentified.

### Immunocytochemistry

To localize and quantitate the increased GAP-43 expression, immunocytochemistry was performed on decalcified tissue sections. In a previous study

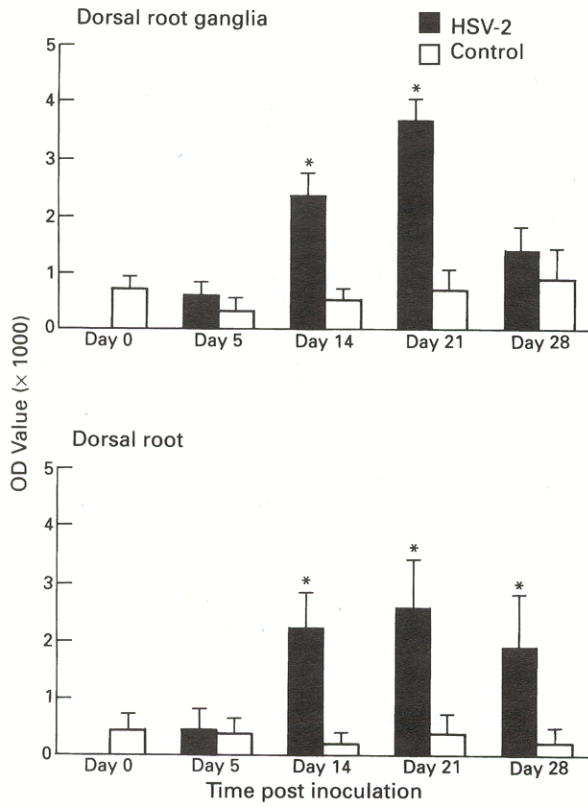


**Figure 1** Western analysis of GAP-43 (43 kd band, arrow) immunoreactivity in L4 and L5 dorsal roots. Increased immunoreactivity is seen 14 days following axotomy (D14-AXOTOMY) when compared to non-axotomized animals (CONTROL). GAP-43 immunoreactivity at both 14 and 28 days following HSV-2 inoculation (D14-HSV, D28-HSV), is greater than in control animals or mock-infected roots (D14-SHAM, D28-SHAM).

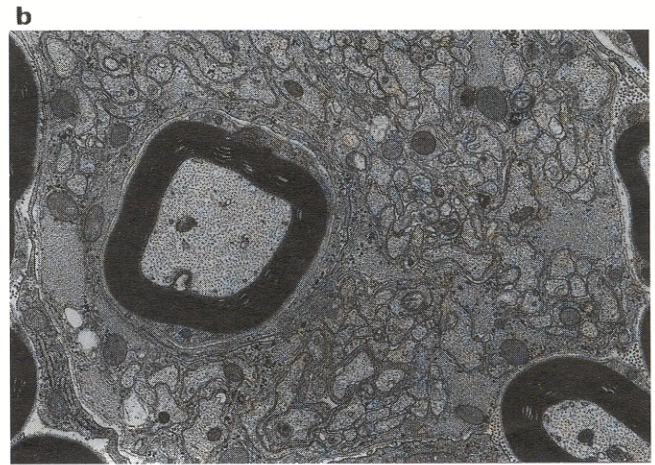
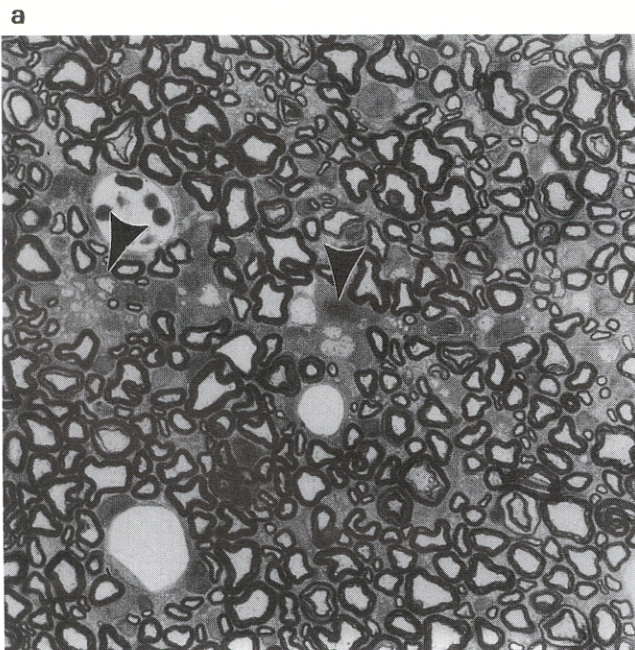


**Figure 2** GAP-43 immunoreacted section of L4 DRG (right side of pictures) and dorsal root (left side of pictures) 14 days following unilateral HSV-2 inoculation of the footpad in the same mouse. (a) Ipsilateral infected side. (b) Contralateral uninfected side. GAP-43 is seen as a dark brown immunoprecipitate. Note the increase in the number and intensity of GAP-43 antigen-positive cell bodies in DRG as well as the increase in antigen in the dorsal root on the infected side. Counterstained with H&E. Magnification = 210 $\times$ .





**Figure 3** Histograms showing relative levels of GAP-43 in ipsilateral infected (solid bars) and contralateral uninfected (white bars) DRG and dorsal roots 0 (uninoculated control) 5, 14 and 28 days following footpad inoculation (x-axis). NIH image was used to determine optical density (OD) values (y-axis; mean OD  $\pm$  SE). GAP-43 antigen is increased 2 weeks following inoculation in both DRG and dorsal roots and remains elevated 1 mo later in dorsal roots. \* indicates statistical significance at  $P < 0.05$  from both contralateral uninfected side and uninoculated controls (0 dpi).



**Figure 4** (a) Toluidine blue stained semi-thin section of dorsal root 4 weeks following unilateral HSV-2 footpad inoculation. Note the unmyelinated patchy areas (arrowheads) and the presence of inflammatory cells. Magnification = 740 $\times$ . (b) EM of one of the patchy unmyelinated areas in HSV-2 infected dorsal root 4 weeks following inoculation. Note the large numbers of abutting neurites and the lack of Schwann cell cytoplasm envelopment. Magnification = 13,200 $\times$ . (c) Uninfected root showing the characteristic Schwann cell with many unensheathed fibers embedded in its cytoplasm. Even in normal mouse dorsal root, however, touching neurites can be found (arrows). Magnification = 17,600 $\times$ .

decalcification was not found to affect antigen detection (Henken and Martin, 1993), while allowing accurate preservation of anatomical relationships and direct comparisons between paired ganglia of the same animal. In Figure 2, an example of DRG and dorsal roots, immunoreacted for GAP-43 antigen and counterstained with H&E, is seen 14 dpi. There is increased GAP-43 immunoreactivity in DRG perikarya and in the dorsal root ipsilateral to the inoculated footpad (Figure 2a), when compared to the DRG and dorsal root of the contralateral uninoculated side (Figure 2b) of the same mouse. Unmanipulated mice (not shown) show similar numbers and intensity of GAP-43 immunoreactivity as the contralateral uninoculated side. The prolonged inflammatory response and ganglionitis evident in the infected DRG and dorsal root and the apparent neuronal loss is similar to that analyzed in mice DRG 28 dpi in a previous study (Henken *et al*, 1993). HSV-2 antigen was only found in DRG 5 dpi (not shown); no antigen was seen at 2 weeks or 1 month following inoculation. This result is in agreement with previous studies on HSV-2, strain MS antigen localization following footpad inoculation (Henken and Martin, 1991; Henken and Martin, 1992a).

To determine the relative quantity of GAP-43 in tissue sections, GAP-43 in non-counterstained DRG and in dorsal roots was analyzed (Figure 3). In the DRG (Figure 3a), there is no difference in GAP-43 immunoreactivity at 5 dpi. By 14 dpi there is a 6-fold ( $P < 0.05$ ) increase in the amount of antigen in the DRG ipsilateral to the inoculated footpad, when compared to either the DRG from the contralateral uninoculated side or the DRG of uninfected mice (0 dpi). Immunoreactivity approaches control levels at 28 dpi, and is not significantly different from the contralateral side.

In the dorsal roots (Figure 3b), no difference is seen at 5 dpi. By 14 dpi an 8-fold increase ( $P < 0.05$ ) is seen in dorsal roots ipsilateral to the HSV-inoculated footpad when compared to either the roots of the contralateral uninoculated side or the roots of unmanipulated controls (0 dpi). This difference is maintained 28 dpi ( $P < 0.5$ ).

#### Electron microscopy

A representative toluidine blue stained semithin section of dorsal root from a mouse which received a unilateral HSV footpad inoculation 1 month earlier is presented in Figure 4a. Patchy non-myelinated regions (arrowheads) containing myelin sheath remnants, several large unmyelinated or thinly myelinated axons, and a few inflammatory cells are present. Electron microscopic examination of one of these numerous non-myelinated regions (Figure 4b) reveals a large bundle ( $> 100$ ) of small caliber unmyelinated fibers lacking ensheathment by Schwann processes. Neurites directly abut each other with no interspersed cytoplasmic processes.

This appearance resembles that seen during nerve development (Peters *et al*, 1991) and regeneration (Tanaka and Webster, 1991). For comparison, an equivalent region through an uninfected dorsal root is presented (Figure 4c). Many small unmyelinated fibers are embedded in Schwann cell cytoplasm and for the most part, are separated by cytoplasm. However, even in normal mouse dorsal roots, unmyelinated axons can be found touching with no intervening cytoplasm. Bundles containing large numbers of neurites ( $> 100$ ) were never seen.

#### Discussion

We have demonstrated by Western blotting and quantitative immunochemistry that GAP-43 is induced in mouse spinal ganglia and roots following footpad inoculation of HSV-2 when compared with mock-infected and control animals. GAP-43 expression correlates with periods of axonal elongation (Skene, 1989) and is greatest in the developing nervous system and becomes down-regulated in the adult (Jacobson *et al*, 1986; Basi *et al*, 1987). Although low levels of GAP-43 persist in many neurons, including spinal sensory neurons (Benowitz *et al*, 1988; Stewart *et al*, 1992), it is rapidly re-induced during regeneration (Skene and Willard, 1981a, b; Verhaagen *et al*, 1986; Tetzlaff *et al*, 1989; Van der Zee *et al*, 1989; Doster *et al*, 1991) or collateral sprouting (Benowitz *et al*, 1990). Previous studies have documented selective neuropeptide and enzyme alterations in sensory neurons following HSV-2 infection (Henken and Martin, 1992a,b; Tenser, 1991) that are consistent with those seen in classical axotomy-induced regeneration models (Tenser, 1985; Hökfelt *et al*, 1987; Villar *et al*, 1989, 1991). Our demonstration of GAP-43 induction in DRG in this model supports the hypothesis that one of the long-term neurobiological effects following HSV infection in spinal ganglia is a sprouting response. Furthermore, our ultrastructural demonstration of large bundles of unmyelinated neurites in dorsal roots 2 weeks and 1 month following HSV infection is clear evidence of centrally-directed sprouting by spinal ganglion neurons. Although it is not yet clear whether neuronal sprouting is a direct or indirect effect of HSV-2 infection, the delayed onset of the response, as well as the delayed onset of neuropeptide (Henken and Martin, 1992a,b) and enzyme (Tenser, 1991) changes, suggest that the effect may be a secondary one, possibly induced by the neuronal loss and/or immune response.

Albeit GAP-43 is clearly induced in DRG perikarya in this model, its discreet localization in the dorsal root has yet to be determined. The GAP-43 measured in dorsal roots could be present in central axons of sensory neurons (Woolf *et al*, 1990; Coggeshall *et al*, 1991; Schreyer and Skene, 1991) or in non-myelin-forming Schwann cells (Curtis *et al*, 1992). Preliminary evidence from GAP-43



immunoreacted 1  $\mu\text{m}$  epon embedded roots, suggests that both cells are stained in HSV-infected roots. The low level of GAP-43 in mock-infected tissues may result from damage to nociceptive terminals in the skin (Cliffer *et al*, 1993).

This virus-induced sprouting model provides an interesting alternative for examination of axon growth regulation in sensory neurons when compared to peripheral axotomy paradigms. One distinct feature of this model is the induction of an inflammatory response in infected ganglia (Gebhardt and Hill, 1988, 1990) which may enhance sprouting. Injection of bacteria or macrophages into DRG has previously been shown to enhance regeneration of central sensory afferent fibers following axotomy (Lu and Richardson, 1991). One could test the hypothesis that the inflammatory infiltrate produces cytokines which provide a nurturing environment for nerve elongation.

We have not yet determined whether latently-infected or uninfected neurons are sprouting in this model. Our previous evidence indicates that HSV infection can result in severe neuronal loss (Henken *et al*, 1993), which may in turn stimulate collateral sprouting of the remaining DRG neurons, similar to lesion-induced sprouting in the hippocampus (Benowitz *et al*, 1990). Alternatively, infected neurons regenerating their peripheral axons may also undergo central sprouting, as has been shown during axotomy-induced regeneration (Coggeshall *et al*, 1991; Woolf *et al*, 1992). To begin to address this issue, we are currently assessing whether the sprouting neurons are latently infected with HSV.

Finally, the severe ganglionitis, the sprouting response, and the potential central reorganization (Woolf *et al*, 1992) that occurs following HSV infection may provide insights into the underlying mechanism of post-herpetic neuralgia, a clinical syndrome caused by reactivation of another member of the herpes family, varicella zoster virus.

## Materials and methods

Female BALB/c mice, 25–30 g, approximately 6 weeks old, obtained from NCI-Frederick Md. Cancer Research Facility, were used for all experiments. Animals were cared for according to Manual Issuance 3040-2 of the NIH Guide for Use of Animals in Intramural Research.

### Virus stock preparation

HSV-2, strain MS was grown in Vero cells using Eagle's minimal essential medium containing 10% fetal calf serum (MEM-FCS). When cytopathic changes were evident in 90–100% of the monolayer, infected cells were scraped from flasks. Cell suspensions were freeze-thawed, sonicated and centrifuged at 2000 rpm for 10 min. Supernatants were removed and aliquots were stored at  $-70^{\circ}\text{C}$ . Virus stocks were titered in a standard plaque assay. The

virus stock titer was  $9.3 \times 10^6$  plaque forming units (pfu)  $\text{ml}^{-1}$ .

### Footpad inoculation and sciatic nerve axotomy

Following induction by methoxyflurane, mice were deeply anesthetized by an intraperitoneal injection of 3.5% chloral hydrate ( $350 \text{ mg kg}^{-1}$ ). A 10  $\mu\text{l}$  droplet of MS ( $9 \times 10^4$  pfu  $10 \mu\text{l}^{-1}$ ) strain of HSV-2 virus of MEM-FCS was placed on the hind footpad(s) and 50 pinpricks were made through the droplet with a 26 gauge needle.

For Western blot analysis, 30 mice were bilaterally inoculated with HSV-2 as described above, while 30 other mice had bilateral footpad inoculations with MEM-FCS and served as controls (mock-infected). Ten unmanipulated mice served as intact controls; in 10 additional mice, the sciatic nerve was exposed under anesthesia and cut at mid-thigh level. For immunocytochemistry, a total of 30 mice were studied. Five mice were uninfected controls, 20 mice were inoculated with HSV-2 on their right hind footpad, while the contralateral footpad was left intact and served as an internal control and five other mice received a right sciatic nerve axotomy. To examine ultrastructural alterations in dorsal roots, four mice had right hind footpad inoculations with HSV-2. The contralateral uninoculated side served as an internal control while two additional unmanipulated mice provided intact controls.

### Western blotting

Fourth and fifth lumbar (L4 and L5) dorsal roots were dissected and pooled from mice at 0 (uninoculated), 5, 14 and 28 days dpi, as well as from mice 14 days following sciatic nerve axotomy. Tissues were homogenized directly in 2% SDS in PBS, and protein concentrations were estimated (Bio-Rad). Homogenates were diluted 1:1 with 2 $\times$  sample buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 10%  $\beta$ -mercaptoethanol) and boiled for 5 min. Preparations of 30  $\mu\text{g}$  protein were subjected to 10% SDS-PAGE. Separated proteins were electrophoretically transferred onto nitrocellulose paper. The blots were reacted with a 1:1000 dilution of rabbit anti-GAP-43 (Curtis *et al*, 1991) at  $4^{\circ}\text{C}$ . Blots were treated with biotinylated goat anti-rabbit IgG and avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit, Vector Labs, Burlingame, CA) according to the manufacturers' specifications and bands were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA).

### Immunocytochemistry

Five, 14, 21, or 28 dpi, mice were anesthetized with methoxyflurane and perfused intracardially with 20 ml 10% formalin in 0.1 M phosphate buffered saline, pH = 7.4 (PBS). For each experiment, five age-matched, uninoculated mice were prepared in a similar manner (0 dpi).

The vertebral column (encasing the lumbosacral spinal cord and ganglia) was dissected free, placed in fixative for an additional 5–7 days, decalcified for 6 days in saturated EDTA solution (disodium salt, Sigma), rinsed 3x in PBS, and stored in 70% ethanol (Henken and Martin, 1993). The tissue was then dehydrated and embedded in paraffin. Sets of longitudinal sections (7  $\mu$ m) were collected on organosilane-treated slides or poly-L-lysine coated slides (Statpath, Riderwood, MD), and deparaffinized.

The first slide of each set was stained with hematoxylin and eosin, the second immunoreacted for HSV-2 antigen, and the third and fourth immunoreacted for GAP-43 antigen. One set of GAP-43 immunoreacted slides was lightly counterstained with hematoxylin, to aid in visualization of non-labeled neurons. The second series was not counterstained and was used in obtaining quantitative data.

Rabbit antisera to GAP-43 (Curtis *et al*, 1991) and HSV-2 (Dako, Santa Barbara, CA) were used at a dilution of 1:1000. In preliminary studies examining antibody dilutions on paraffin embedded tissues, we found that a dilution of 1:1000 resulted in specific labelling which could be clearly distinguished from background. The avidin-biotin method of Hsu *et al* (1981a,b) was employed to detect antigen. Briefly, mounted sections were hydrated, blocked in BSA, reacted overnight with primary antibody, and treated with biotinylated goat antirabbit IgG and avidin-biotinylated horse-radish peroxidase complex (Vectastain ABC Kit, Vector Labs, Burlingame, CA) as specified by the manufacturer. DAB was used to visualize the immunoreactive cells. Tissues were examined at the light microscopic level and antigen-containing structures were identified. As a control for anti-serum specificity, primary antiserum was omitted from selected adjacent sections.

The series of uncounterstained GAP-43 immunoreacted tissues were analyzed both on Macintosh (National Technical Information Service, Springfield, VA, USA) (NIH image) and Bioquant (R & M Biometrics Inc, Nashville, TN, USA) image

analysis systems. Briefly, a constant background threshold was determined from uninfected control sections and maintained throughout the data collection procedure. For each section a defined area of L4 and L5 DRG and proximal dorsal root ipsilateral to the inoculated footpad was analyzed and compared to the equivalent area on the contralateral side. The immunoreactive area and the density of the immunoreactive area within the scanned region were determined. The optical density (OD) value was calculated and background values were automatically subtracted during analysis. The OD value for the ipsilateral infected DRG and its dorsal root was directly compared to the OD value for the contralateral uninfected DRG and its dorsal root within each tissue section. For each average OD value ( $\pm$  SE), at least eight sections from each of four mice were analyzed.

#### Electron microscopy (EM)

Anesthetized mice 0, 14 and 28 dpi ( $n = 2$  for each group) were perfused intracardially with 100 ml of fixative (4% paraformaldehyde, 15% (v v<sup>-1</sup>) saturated picric acid, 0.08% glutaraldehyde, 0.1 M PBS (Somogyi and Takagi, 1982). Right and left L4 and L5 dorsal roots were placed in fresh fixative overnight and post-fixed in 1% osmium tetroxide for 3 h the following day. Following dehydration, all tissues were individually embedded in Epon.

Semi-thin sections, stained with toluidine blue were examined from blocks of both ipsilateral (to inoculated footpad) and contralateral dorsal roots. Thin sections were prepared from selected blocks, stained with uranyl acetate and lead citrate and examined with a Phillips (Eindhoven, The Netherlands) 410 electron microscope.

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