

Selective Spread of Herpes Simplex Virus in the Central Nervous System after Ocular Inoculation

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The spread of herpes simplex virus (HSV) was studied in the mouse central nervous system (CNS) after ocular inoculation. Sites of active viral replication in the CNS were identified by autoradiographic localization of neuronal uptake of tritiated thymidine. Labeled neurons were first noted in the CNS at 4 days postinoculation in the Edinger-Westphal nucleus, ipsilateral spinal trigeminal nucleus, pars caudalis, pars interpolaris, and ipsilateral dorsal horn of the rostral cervical spinal cord. By 5 days postinoculation, additional sites of labeling included the seventh nerve nucleus, nucleus locus coeruleus, and the nuclei raphe magnus and raphe pallidus. None of these sites are contiguous to nuclei infected at 4 days, but all are synaptically related to these nuclei. By 7 days postinoculation, no new foci of labeled cells were noted in the brain stem, but labeled neurons were noted in the amygdala, hippocampus, and somatosensory cortex. Neurons in both the amygdala and hippocampus receive axonal projections from the locus coeruleus. On the basis of these findings, we conclude that the spread of HSV in the CNS after intracameral inoculation is not diffuse but is restricted to a small number of noncontiguous foci in the brain stem and cortex which become infected in a sequential fashion. Since these regions are synaptically related, the principal route of the spread of HSV in the CNS after ocular infection appears to be along axons, presumably via axonal transport rather than by local spread.

Infection of the central nervous system (CNS) is common in animal models of herpetic eye disease, and the route of spread of herpes simplex virus (HSV) from the anterior segment of the eye along the trigeminal nerve and into the brain stem has been well described elsewhere (1, 5, 11, 22). Much less is known, however, about the specific location of brain stem neurons that become initially infected after ocular inoculation or about the subsequent routes of viral spread in the CNS.

The brain stem trigeminal complex appears to be the initial site of viral infection within the CNS after corneal inoculation with HSV. This was originally demonstrated histologically by Goodpasture and Teague (5) and later confirmed through the use of electron microscopy (1), immunocytochemistry (11), and in situ hybridization (22). However, in each of these more recent studies, different portions of the trigeminal complex have been reported to be infected. Baringer and Griffith (1) reported viral capsids within the descending trigeminal tracts; Knotts and co-workers (11) described viral antigens in the principal sensory nucleus, descending tracts, and spinal nuclei; and Stroop and co-workers (22) found evidence of viral replication within neurons of the main sensory nucleus, divisions of the spinal nucleus, and mesencephalic nucleus. Furthermore, although each of these groups of investigators, as well as others, commented on the subsequent spread of the virus to additional CNS regions (3, 19, 23), the precise locations of these sites have remained ill defined.

We have recently described a new technique for identifying HSV-infected neurons by using systemically administered [³H]thymidine (15). Mature neurons are postmitotic and fail to be labeled by this marker, but neurons that are actively infected with HSV readily incorporate the [³H]thymidine and can be easily identified by autoradiography. The advantages of this technique for the identification

of HSV-infected neurons include technical simplicity, a high degree of reproducibility, and excellent tissue preservation.

In the present study, we have utilized this technique not only to assess the location of brain stem neurons that initially become infected with HSV after inoculation of the anterior chamber of the eye but also to study the sequential spread of the virus within the brain stem and to other areas of the CNS. We have chosen an intracameral site of inoculation in order to expose the cornea to HSV yet confine the peripheral spread of the infectious virus as much as possible. Our findings suggest that the spread of HSV within the brain stem trigeminal system as well as to other brain stem nuclei primarily occurs along a limited number of synaptically related neuronal pathways. These findings suggest that the principal route of the spread of HSV in the CNS is trans-neuronal via intra-axonal transport rather than local cell-to-cell infection.

MATERIALS AND METHODS

ICR mice, 8 to 12 weeks old, were anesthetized by an intramuscular injection of ketamine-xylazine (5:1) followed by topical corneal administration of proparacaine. With microscopic guidance, a limbal paracentesis was performed with a 27-gauge needle, and a hand-pulled glass micropipette was inserted into the anterior chamber of the left eye of each animal. Through the pipette, 100 PFU of McKrae strain HSV in 1 μ l of minimal essential medium was injected. The pipette was then removed from the eye, and the cornea was treated with 1% atropine. Control animals were injected with 1 μ l of sterile medium.

Up to 7 days after viral inoculation, mice were injected intraperitoneally with 350 to 400 μ Ci of [³H]thymidine (specific activity, 211 μ Ci/mg) and sacrificed 2 h later under deep pentobarbital anesthesia by cardiac perfusion with phosphate-buffered saline solution (pH 7.3). Tissues were then fixed by cardiac perfusion with 3% glutaraldehyde in phosphate buffer (pH 7.3), and the brains were carefully dissected

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and placed overnight in phosphate-buffered glutaraldehyde at 4°C. The tissues were dehydrated through multiple changes of methylcellosolve (ethylene glycol monomethyl ether) and embedded in glycol methacrylate. Serial tissue sections were cut at a thickness of 3 μ m, and every 17th section was mounted on a glass microscope slide and dipped for autoradiography. After a 4-week exposure, the slides were developed, counterstained with thionine, and evaluated by light microscopy.

A total of 22 mice were used in this study. Two of these mice were inoculated with sterile medium and served as controls. Of the 20 mice inoculated with HSV, 2 each were sacrificed at 1 and 2 days postinoculation, 4 each were sacrificed at 3 and 4 days postinoculation, 5 were sacrificed 5 days postinoculation, and 3 were sacrificed 7 days postinoculation. All procedures with the animals adhered to the guidelines of the University of California, San Francisco, Committee on Animal Research.

RESULTS

Anterior chamber inoculation of ICR mice with 100 PFU of McKrae strain HSV uniformly resulted in signs of encephalitic infection. By 6 days postinoculation, all infected animals displayed behavioral changes, including marked lethargy, irritability, and asymmetric weakness. Animals injected with the sterile medium remained healthy.

Light microscopic evaluation of tissue sections prepared for autoradiography revealed silver grain labeling over distinct CNS neuron populations in all animals sacrificed at 4 days or later after inoculation with HSV. The sites of neuronal labeling are summarized in Fig. 1. Labeled neurons frequently, but not always, demonstrated the characteristic features of an HSV-infected cell, including clumped chromatin, a pale nucleus, and vacuolated cytoplasm. No labeled neurons were seen in animals sacrificed at 1, 2, or 3 days after inoculation with HSV. Furthermore, no labeling of CNS neurons was seen in animals injected with the sterile medium.

Four days after anterior chamber inoculation with HSV, radioactively labeled neurons were first observed in the CNS. Labeled cells were sparse, however, and mainly limited to the Edinger-Westphal (EW) nucleus and the ipsilateral spinal trigeminal nucleus at the level of the pars interparalis, pars caudalis, and rostral cervical spinal cord (Fig. 2). In two animals, a few labeled neurons were also noted in the principal oculomotor nucleus. The labeled cells were consistently located ipsilateral to the site of injection, but a few labeled oculomotor neurons were also noted contralateral to the site of injection along the medial aspect of the nucleus.

By 5 days after anterior chamber inoculation, labeling remained limited to the brain stem but was more extensively distributed than at 4 days. Labeled neurons could still be found in the spinal trigeminal nucleus, the EW nucleus, and the oculomotor nerve nucleus, but the number of identifiable labeled neurons was less than that seen at 4 days, and extensive tissue destruction and an inflammatory cell infiltrate were seen in these areas (Fig. 3). In contrast, prominently labeled neurons with minimal associated inflammation were consistently found in the nucleus raphe magnus, nucleus raphe pallidus, nucleus locus coeruleus, and facial nerve nucleus at 5 days postinoculation (Fig. 4). All of these nuclei either project to or receive projections from the spinal nucleus of the trigeminal complex. Labeling of the nucleus locus coeruleus was bilateral and extensive. Adjacent neu-

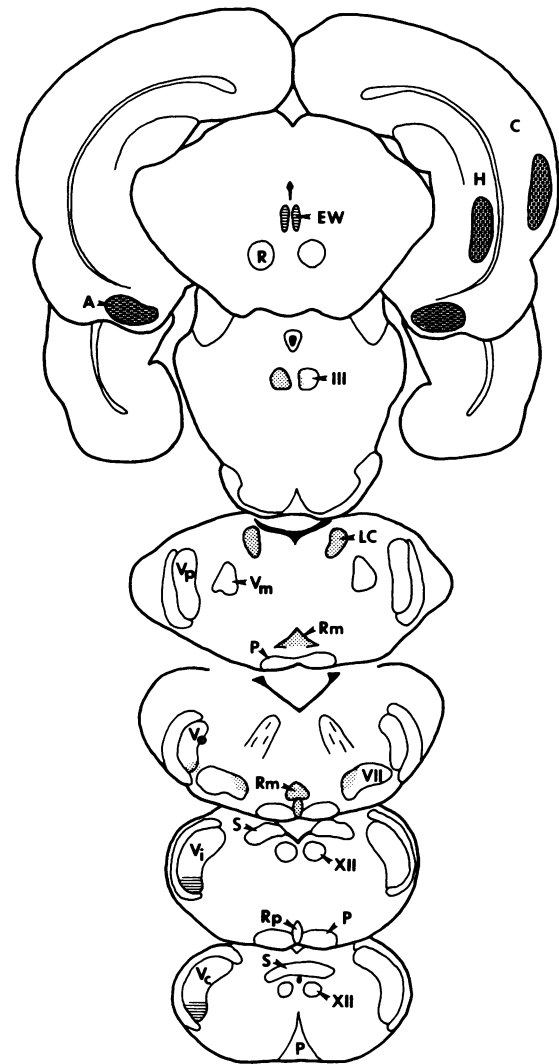


FIG. 1. Summary diagram of the sites of active HSV infections in the CNS of mice after intracameral injections of HSV. Striped regions represent nuclei first labeled at 4 days postinoculation; stippled areas represent sites where labeling was first found at 5 days postinoculation; and circles represent sites where labeling was first found at 7 days postinoculation. A, Amygdala complex; C, cerebral cortex; H, hippocampal formation; LC, nucleus locus coeruleus; S, nucleus solitarius; R, red nucleus; V_c, nucleus caudalis; V_i, nucleus interparalis; V_o, nucleus oralis; V_m, main sensory nucleus; Rm, nucleus raphe magnus; Rp, nucleus raphe pallidus; P, pyramidal fibers; III, oculomotor nucleus; VII, facial nerve nucleus; XII, hypoglossal nucleus.

rons of the mesencephalic nucleus, however, were unlabeled. Labeling of neurons of the facial nerve nucleus was also bilateral but was restricted to a small population of neurons in the dorsomedial portion of the nucleus. At this time, a few labeled neurons were also seen in the reticular formation and main sensory nucleus of the trigeminal complex.

In animals sacrificed 7 days after ocular inoculation, no new foci of labeling in the brain stem were noted. However, neuronal labeling in the forebrain was noted in the hippocampus, amygdala, and, in one animal, a small region of the somatosensory cortex. Labeled neurons were found bilaterally in the amygdala, but only a small number of tightly

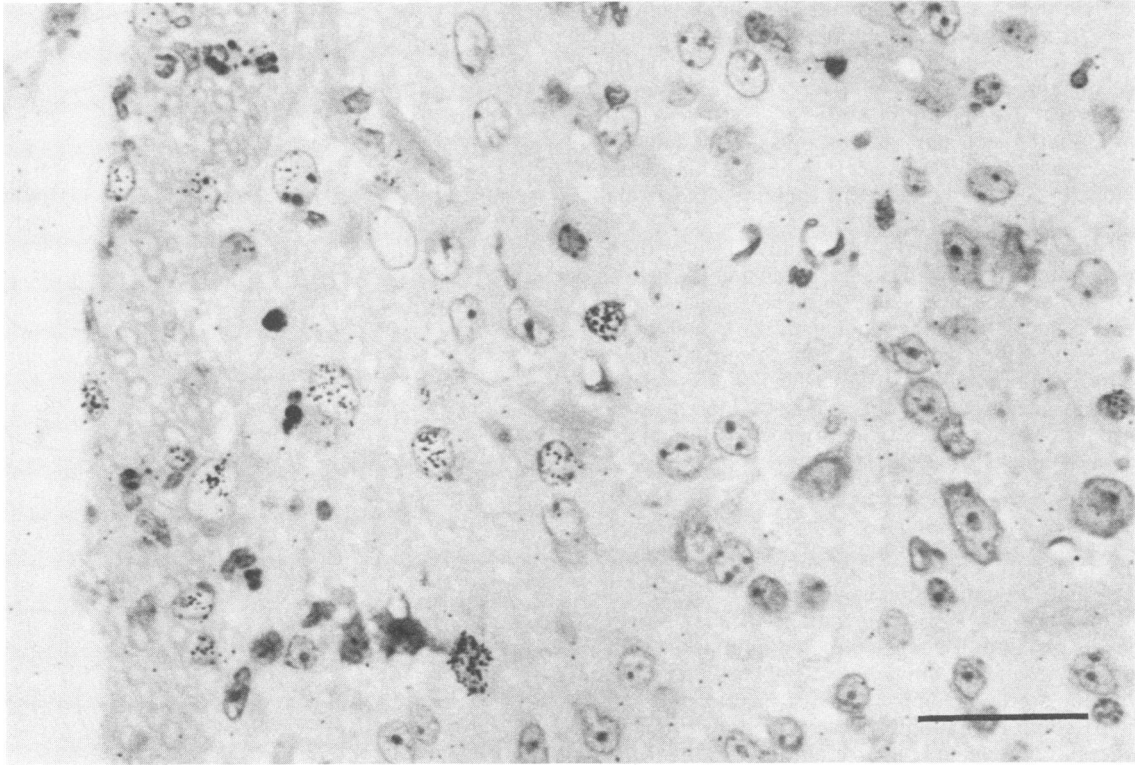


FIG. 2. Examples of labeled neurons of the nucleus pars caudalis of the trigeminal complex. Active viral replication was noted as early as 4 days postinoculation in this region. Bar = 40 μ m.

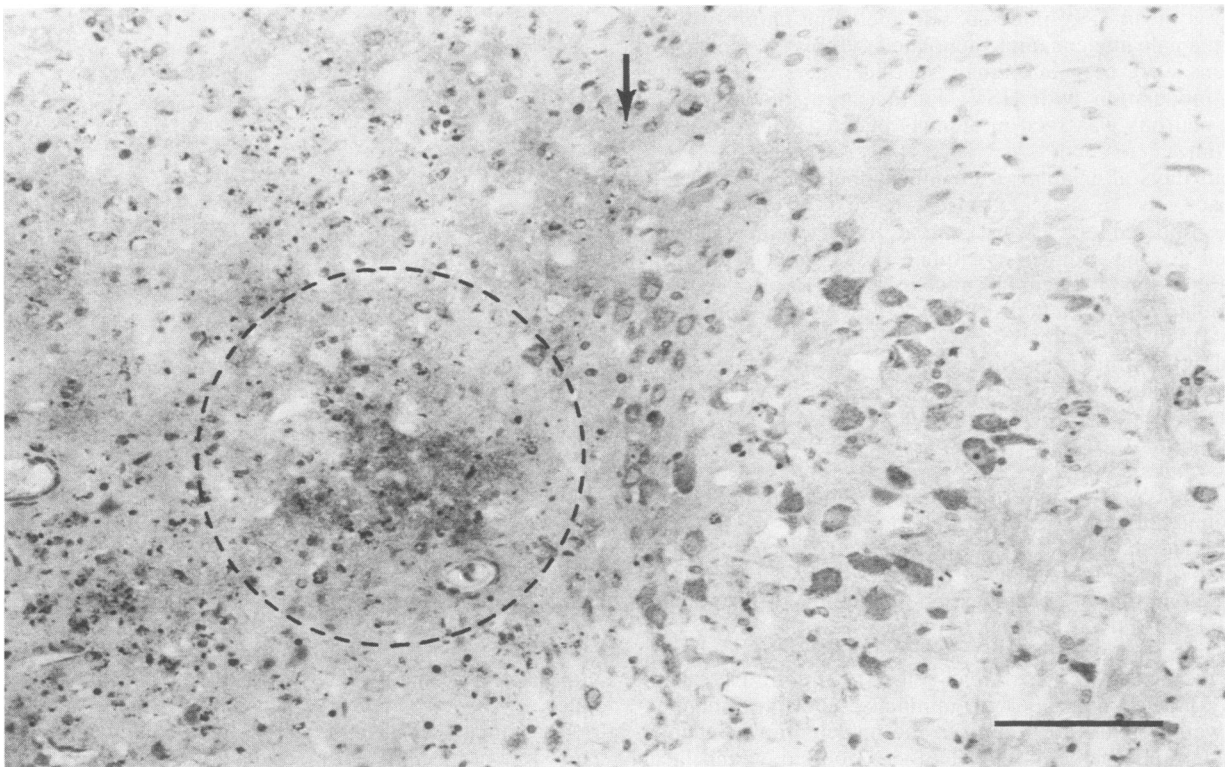


FIG. 3. Light micrograph of the oculomotor nuclei 5 days after inoculation with HSV. Destruction and inflammatory cell infiltrate were seen on the affected side (encircled area). The arrow marks the midline. A similar pathology was seen in the EW nucleus, nucleus pars caudalis, and dorsal horn of the cervical spinal cord at 5 days postinoculation. In brain stem nuclei exhibiting these pathological changes, the degree of radioactive labeling was markedly attenuated, which possibly reflects the loss of viable neurons capable of sustaining an active infection with HSV. Bar = 0.25 μ m.

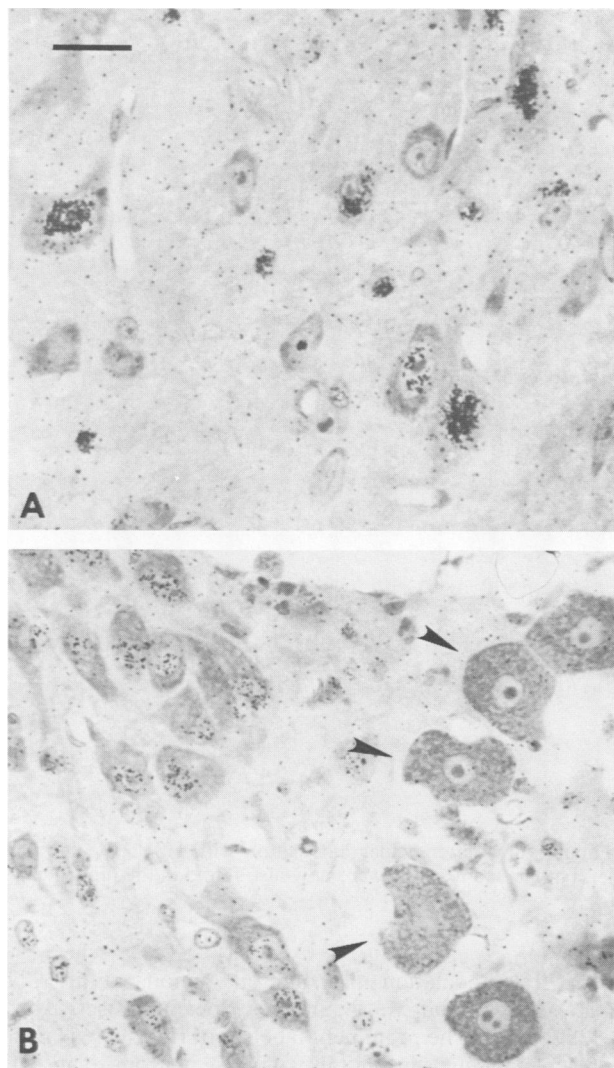


FIG. 4. Light micrographs of labeled neurons in the CNS at 5 days postinoculation. Motor neurons of the facial nerve nucleus (A) and the nucleus locus coeruleus (B) were labeled bilaterally. Note the absence of labeling in cell bodies of mesencephalic V nucleus (arrows). Bar = 25 μ m.

clustered pyramidal cells were found in the contralateral CA1 region of the hippocampus (Fig. 5). Cortical labeling was similarly tightly clustered in a small region of somatosensory cortex directly overlying the labeled portion of the hippocampus. Cortical tissues surrounding the hippocampus and somatosensory cortices, however, were free of label and inflammatory cells.

Labeled glial cells were also observed in the CNS of infected mice but were less prominently labeled than neurons. The distribution of labeled glia largely reflected the distribution of labeled neurons, but with a temporal delay of 24 to 48 h. Since glia are capable of cell division in response to inflammatory stimuli, it is unclear whether labeling of these cells reflects active viral infection or simply a mitotic response to local neuronal necrosis and viral antigen.

DISCUSSION

In addition to its utility for studying HSV-infected neurons in the peripheral nervous system, the systemic administra-

tion of [3 H]thymidine appears to be a simple and effective means of labeling CNS neurons that are infected with actively replicating HSV. In this study, we have used this technique to follow the spread of HSV within the CNS after anterior chamber inoculation. We have chosen to study the results of a low-titer inoculation into the anterior chamber in order to confine the site of peripheral viral infection and thus simplify the pattern of CNS spread. Our findings suggest that infection of the CNS by HSV after ocular inoculation is not diffuse but is limited to a few brain stem nuclei and progresses primarily by axonal transport. Even at 7 days postinoculation, when animals were in a premoribund state, the distribution of infected neurons in the CNS was patchy and limited to a small number of foci of infection.

A more widespread distribution of HSV in the CNS has been reported after corneal inoculation (4, 22, 23). It is likely that this reflects the use of a larger viral inoculum and a less restricted site of inoculation in these studies. The viral inoculum used in these earlier studies was at least 10^4 times greater than that used in our protocol and was topically applied to the cornea. Inoculations by this approach readily infect ocular adnexal structures, including the nasal lacrimal drainage system (23). It is thus possible that the somewhat more diffuse CNS spread of HSV that has previously been reported to follow corneal inoculation is due to more extensive peripheral infection.

In the present study, infected neurons were first seen in the CNS 4 days after anterior chamber inoculation with HSV in the EW nucleus, as well as in the spinal trigeminal nucleus at the levels of the pars caudalis, pars interpolaris, and dorsal horn of the rostral cervical spinal cord. The early localization of labeled neurons to these nuclei suggests that these are the principal portals of entry of the virus into the CNS. It would thus appear that both parasympathetic nerves and primary sensory neurons are responsible for the transport of HSV to the CNS in this model of herpetic encephalitis. Dolivo and co-workers (13, 16) have previously described infection of the EW nucleus after intraocular injection of the rat with either rabies virus or herpes virus suis, but we are unaware of any published description of the transport of HSV along a parasympathetic pathway. Since the anterior segment of the eye may receive direct axonal projections from the EW nucleus (7, 18, 20, 27), it is possible that the early labeling of neurons in the EW nucleus after anterior segment inoculation with HSV was due to axonal transport of the virus along a direct, nonsynaptic pathway. These findings also raise the possibility that latent infection of the EW nucleus may serve as a viral reservoir for recurrent herpetic iridocyclitis.

An unexpected finding at 4 days postinoculation was the presence of infected neurons in the oculomotor nucleus. Infection of oculomotor neurons after ocular inoculation has not been previously reported with HSV. It is possible that these neurons became infected by contiguous spread from infected neurons of the EW nucleus. Another possibility is that there was leakage of the virus at the site of inoculation, with resultant infection of the motor neurons that supply the extraocular muscles. Retrograde transport of HSV into the CNS by motor neurons has been previously described (17, 24, 25), and infection of either the levator palpebrae or the superior rectus muscles would account for the bilateral, albeit asymmetric, distribution.

The limited involvement of the trigeminal sensory complex at 4 days postinoculation is consistent with the retrograde tracing studies of Marfurt and Del Toro (14), who have demonstrated that sensory neurons of the rat cornea project

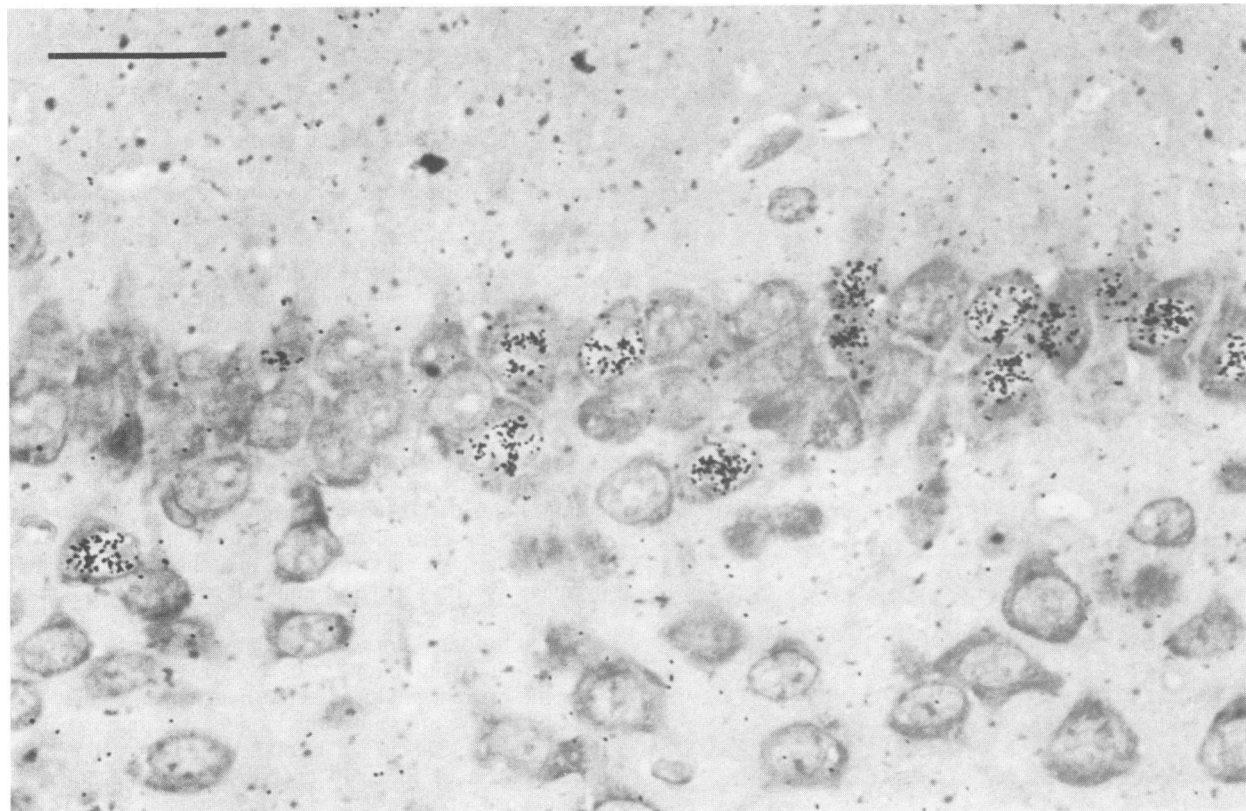


FIG. 5. Light micrograph of labeled pyramidal neurons in the hippocampus 7 days after ocular inoculation with HSV. By this time, neuronal labeling was still restricted to a limited number of brain stem nuclei, but labeled neurons were now also found in the hippocampus and amygdala, which indicates cortical spread of infection. Bar = 60 μ m.

primarily to the nucleus pars caudalis and pars interparialis of the trigeminal complex and to the dorsal horn of the high cervical spinal cord. Although other investigators have reported infected neurons in the principal sensory nucleus after ocular inoculation (4, 14, 22), we detected no labeling of these neurons until 5 days postinoculation. In light of the known axonal projections from the principal sensory nucleus to the nucleus pars caudalis (6) as well as the retrograde tracing studies of Marfurt and Del Toro (14) that failed to demonstrate connections from the cornea to the main sensory nucleus of the trigeminal complex, we suggest that the spread of HSV to the main sensory nucleus probably occurs by transneuronal transfer in the nucleus pars caudalis to axons that transport HSV in a retrograde direction to main sensory neurons. In a similar fashion, axonal transport was probably responsible for the spread of the virus to the neurons of the midline raphe nuclei, nucleus locus coeruleus, and VII nerve nuclei by 5 days postinoculation. These nuclei are not contiguous with those areas infected by the virus at 4 days, but their constituent neurons are known to be synaptically related to cells of the nucleus caudalis (2, 21, 26). These findings are consistent with the work of Kristensson and co-workers (12) and Ugolini et al. (25), who have previously described the axonal transport and transneuronal spread of HSV in the CNS following peripheral inoculation.

The most prominently labeled population of neurons seen on day 5 after ocular inoculation were the neurons of the nucleus locus coeruleus. Infection of these neurons has been reported after inoculation of HSV into the snout (12), hypoglossal nerve (24), and limb nerves (25) as well as after

direct viral inoculation into different regions of the CNS (J. H. McLean and M. T. Shipley, Soc. Neurosci. Abstr. 13:1399, 1987). The prominent labeling of this nucleus might represent a unique susceptibility of these neurons to herpetic infection but may also reflect the extensive branching of the axonal tree that extends from these neurons through the CNS (10). This extensive innervation may provide a route by which the nucleus locus coeruleus could become infected by retrograde axonal transport from any number of infected sites. If the animals were to survive long enough, it might also provide a route for the subsequent diffuse spread of HSV in the CNS by anterograde axonal transport. Although we failed to see diffuse CNS spread of the virus by 7 days in these experiments, we did see spread to the amygdala and hippocampus. Both the amygdala and hippocampus receive projections from the locus coeruleus (8, 9).

Although the mesencephalic nucleus and motor nucleus of V both contribute axons to the trigeminal nerve and are part of the trigeminal brain stem nuclear complex, no radioactive labeling of the neurons of these nuclei was noted in any of our experimental animals. This is in contrast to the findings of Fraser and co-workers (4, 22), who have described latent herpetic infection of the mesencephalic nucleus after corneal inoculation with HSV. One possible explanation for our failure to detect virus in the mesencephalic nucleus might be that infection of these neurons proceeds directly to a latent stage without passing through an active phase of viral DNA replication. Under such circumstances, infected neurons would not be expected to be labeled with [3 H]thymidine.

Alternatively, the relatively restricted nature of our injection may have precluded infection of this nucleus.

In any single CNS focus, [³H]thymidine labeling was first seen in neurons, followed 24 to 48 h later by labeling of nearby glial cells. Thus, even assuming that some of the labeled glial cells represent cells actively infected with HSV, it is unlikely that these cells played a primary role in the spread of HSV in the mouse CNS.

In conclusion, through the use of systemically administered [³H]thymidine, we have studied the sequential spread of active viral replication in the mouse CNS after anterior chamber inoculation with HSV. Our findings suggest that the resultant CNS infection is not diffuse but rather is restricted to a small number of noncontiguous foci in the brain stem and forebrain. Furthermore, the infected areas largely appear to be synaptically related, which suggests that the principal route of spread of infection in the CNS is transneuronal, presumably via axonal transport.

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