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In Vivo Reactivation of Latent Herpes Simplex Virus 1 in Mice Can Occur in the Brain before Occurring in the Trigeminal Ganglion

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ABSTRACT

Herpes simplex virus 1 (HSV-1) establishes latency in neurons of the brains and sensory ganglia of humans and experimentally infected mice. The latent virus can reactivate to cause recurrent infection. Both primary and recurrent infections can induce diseases, such as encephalitis. In humans, the majority of encephalitis cases occur as a recurrent infection. However, in the past, numerous mouse studies documented that viral reactivation occurs efficiently in the ganglion, but extremely rarely in the brain, when assessed *ex vivo* by cultivating minced tissue explants. Here, we compare the brains and the trigeminal ganglia of mice latently infected with HSV-1 (strain 294.1 or McKrae) for levels of viral genomes and *in vivo* reactivation. The numbers of copies of 294.1 and McKrae genomes in the brain stem were significantly greater than those in the trigeminal ganglion. Most importantly, 294.1 and McKrae reactivation was detected in the brain stems earlier than in the trigeminal ganglia of mice treated with hyperthermia to reactivate latent virus *in vivo*. In addition, the brain stem yielded reactivated virus at a high frequency compared with the trigeminal ganglion, especially in mice latently infected with 294.1 after hyperthermia treatment. These results provide evidence that recurrent brain infection can be induced by the reactivation of latent virus in the brain *in situ*.

IMPORTANCE

Herpes simplex virus 1 (HSV-1) establishes latency in neurons of the brains and sensory ganglia of humans and experimentally infected mice. The latent virus can reactivate to cause recurrent infection. In the past, studies of viral reactivation focused on the ganglion, because efficient viral reactivation was detected in the ganglion but not in the brain when assessed *ex vivo* by cultivating mouse tissue explants. In this study, we report that the brain contains more viral genomes than the trigeminal ganglion in latently infected mice. Notably, the brain yields reactivated virus early and efficiently compared with the trigeminal ganglion after mice are stimulated to reactivate latent virus. Our findings raise the potential importance of HSV-1 latent infection and reactivation in the brain.

Herpes simplex virus 1 (HSV-1) infects about 80 to 90% of the human population in the world (1, 2). During infection, the virus replicates productively in peripheral tissues before spreading to replicate in the peripheral sensory ganglia and the central nervous system (CNS) (1, 3). Subsequently, infectious virus is cleared, but some viruses establish latency by depositing their genomes in the neurons of both the peripheral and central nervous system. During latency, latency-associated transcripts are abundantly expressed, whereas the expression of other viral genes is severely repressed. The latent virus can reactivate periodically to cause recurrent infection. Both primary and recurrent infections can induce lesions, mainly in the peripheral tissues and occasionally in the brain to cause encephalitis. HSV-1-induced encephalitis has been the most common cause of sporadic, fatal encephalitis, with an incidence of 1 in 200,000 individuals per year (1). It is associated with 70% mortality in untreated patients and 30% mortality in treated patients (1, 4). Survivors are often left with severe and permanent neurological sequelae, and only 2.5% of all patients regain normal neurological function (1). As 70% of encephalitis cases are reported to be recurrent infections (5), understanding the incidence of recurrent brain infection may help to combat this tragic disease.

The murine model has been used to study HSV-1 reactivation, because the virus establishes latency and then reactivates following stimulation in a way similar to that in humans (6–10). In the past 3 decades, numerous *ex vivo* studies documented that viral reactivation occurs efficiently and consistently in the ganglia but extremely rarely in the CNS (such as the brain) when assessed with a conventional assay by cocultivating minced tissue explants with monolayers of cells that support viral growth (6, 9, 11–13). As latency involves three processes, establishment, maintenance, and reactivation, the failure of latent virus to reactivate from the minced brain explant after cultivation has led to the assumption that the brain may not be a latently infected organ (14). Therefore, it is generally believed that the ganglia are the prime source of reactivated virus. The recurrent brain infection found in animal hosts is likely to be due to the spread of reactivated virus from the ganglia.

Recently, we modified the *ex vivo* reactivation assay by dissociating the mouse CNS explant into single cells, which promoted

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cell survival and resulted in a reactivation frequency of up to 80% for both HSV-1 and HSV-2 in the brain stem (10, 15). This finding shows that the latent virus in the CNS is competent to reactivate in explants. However, this does not prove that the latent virus in the CNS can reactivate *in vivo*. In the present study, by subjecting latently infected mice to hyperthermia in order to reactivate the virus *in vivo*, we found reactivated virus in the brain before finding it in the trigeminal ganglion.

MATERIALS AND METHODS

Cells, viruses, and mice. Vero cells were maintained and propagated according to the instructions of the American Type Culture Collection. Wild-type HSV-1 strains 294.1 (15–18) and McKrae (kindly provided by Donald Coen, Harvard Medical School) were propagated and titrated on Vero cell monolayers. All mouse experiment protocols were approved by the Laboratory Animal Committee of National Cheng Kung University. C57BL/6N mice were obtained from Charles River Laboratories International, Inc. (Wilmington, MA), and maintained in the laboratory animal center of our college.

Infection of mice. Six- to 8-week-old C57BL/6N mice were anesthetized and infected with 1×10^7 to 3×10^7 PFU/eye of strain 294.1 or 3×10^5 PFU/eye of strain McKrae (in 5 to 10 μl) on the right eye following scarification (20 times) of the cornea with a needle. The deaths of infected mice were monitored for 30 days.

Measurement of viral titers or detection of infectious virus in latently infected tissues. Mice were infected with virus on the right eye, so the ipsilateral (right) trigeminal ganglia were used for investigation. Mouse brain stems and trigeminal ganglia were harvested, weighed, combined with 1 ml medium, and frozen at −80°C. The mean weights of one brain stem and one trigeminal ganglion were about 80 and 7 mg, respectively. Tissues were thawed, homogenized, and frozen again. Tissue homogenates were thawed, sonicated, and centrifuged. The resulting samples were titrated for infectious virus by plaque assay on Vero cell monolayers overlaid with medium containing 1.5% methylcellulose for 3 days.

Quantitative real-time PCR. Brain stems and trigeminal ganglia were harvested from infected mice 30 days after infection, frozen, and homogenized in solution with guanidine thiocyanate. DNA was extracted, and the amounts of viral (thymidine kinase) and cellular (adipsin) DNA in samples were quantified by real-time PCR as previously described (10, 15). The quantification standards of viral genomes were prepared by reconstituting known amounts (copies) of HSV-1 genomes with homogenates of tissues from uninfected mice. Known amounts of tissue DNA (in μg) prepared from mouse neural tissues were used as the quantification standards for cellular DNA. The number of copies of viral genomes was normalized to the amount of cellular DNA in the sample and expressed as viral genome copies/tissue or μg of tissue DNA.

Hyperthermia treatment and detection of reactivated virus. Mice infected with virus for 30 days were placed in a restrainer and subjected to 43°C for 10 min in a constant-temperature circulating water bath (19). Then, the mice were gently blotted with paper towels and placed under a warm lamp for 30 min to prevent hypothermia. Mouse brain stems and trigeminal ganglia were collected, placed in tubes with 1 ml medium, and frozen. The tissues were thawed, homogenized, and frozen again. The tissue homogenates were thawed and sonicated. The homogenate of one trigeminal ganglion was combined with 2 ml medium before being plated onto one well of Vero cell monolayers with 4×10^5 cells/well in six-well plates seeded the day before. Brain stem homogenates were centrifuged. The resulting supernatant from one brain stem was combined with 2 ml of medium, and the resulting pellet was resuspended in 6 ml of medium before being plated onto one and two wells of Vero cell monolayers with 6×10^5 cells/well in six-well plates seeded the day before. Cultures were incubated in medium without methylcellulose, and the culture medium was changed after 3 days. The Vero cell monolayers were inspected daily for 6 days for the presence of cytopathic effect. All the cultures were then collected and frozen. The frozen cultures were thawed, sonicated, and plated onto fresh Vero cell monolayers with 2×10^5 cells/well in 12-well plates seeded the day before. The next day, the culture medium was removed, and the Vero cell monolayers were overlaid with medium containing methylcellulose, cultured for 3 more days, and stained with crystal violet to detect plaques. Any well in which viral plaques were detected was scored positive for the specimen assayed.

To assess the sensitivity of this two-step detection assay, we performed reconstitution experiments in which neural tissues from latently infected mice without hyperthermia treatment were placed in 1 ml medium with or without 10 PFU of 294.1 and processed as described above to detect infectious virus. Three brain stem samples and three trigeminal ganglion samples reconstituted without virus failed to show cytopathic effect in the first step (6-day culture) and plaques in the second step. In neural tissue samples reconstituted with virus, all three trigeminal ganglion samples showed cytopathic effect, but all three brain stem samples failed to do so, during the first step. All three brain stem samples showed plaques in the second step. Therefore, the two-step detection assay could detect 10 PFU of HSV-1 in trigeminal ganglion and brain stem samples.

Quantification of reactivated virus by plaque assay. Brain stems and trigeminal ganglia of latently infected mice treated with hyperthermia were collected, placed in tubes with 1 ml medium, and frozen. The tissues were thawed, homogenized, and frozen. The tissue homogenates were thawed and sonicated. The homogenate of one trigeminal ganglion was distributed to nine wells of Vero cell monolayers with 2×10^5 cells/well in 12-well plates seeded the day before. Brain stem homogenates were centrifuged. The resulting supernatant from one brain stem was distributed to nine wells of Vero cell monolayers, and the resulting pellet was resuspended with 300 μl of medium and transferred to four wells of Vero cell monolayers. After 1-h incubation, Vero cell monolayers were overlaid with medium containing methylcellulose, cultured for 4 days, and stained to count plaques. To assess the assay, we performed reconstitution experiments in which neural tissues from latently infected mice without hyperthermia treatment were placed in 1 ml medium with or without 25 PFU of 294.1 and processed as described above. Three brain stem samples and three trigeminal ganglion samples reconstituted without virus failed to show plaques. In three brain stem samples reconstituted with virus, 1, 1, and 3 plaques were detected in the samples, respectively. In three trigeminal ganglion samples reconstituted with virus, 7, 7, and 8 plaques were detected in the samples, respectively.

***Ex vivo* explant assay.** Brain stems and trigeminal ganglia of mice infected with virus for 30 days were harvested to assay for the reactivation of latent virus using the mincing or dissociation method, as previously described (10). Briefly, for the mincing method, tissues were finely chopped. For the dissociation method, the chopped tissues were dissociated into a single-cell suspension using trypsin and collagenase. The chopped or dissociated tissues were plated on Vero cell monolayers to monitor cytopathic effect for 10 days and then subjected to plaque assay for 4 days.

Statistical analyses. Data are expressed as means ± standard errors (SE). For statistical comparison, the numbers of latent genomes were analyzed by a Mann-Whitney U test. Viral reactivation frequencies were analyzed by Fisher's exact test.

RESULTS

More viral genomes are detected in the brain stem than in the trigeminal ganglion in mice latently infected with HSV-1. Few reports have compared the brain and the trigeminal ganglion for HSV-1 reactivation *in vivo*, so this study was undertaken to address the issue using the murine model. Our previous report investigating the *ex vivo* reactivation of several HSV-1 strains in the brains of three mouse strains found the following (15). Among the HSV-1 strains examined, 294.1 and McKrae showed high levels of replication, latent genomes, and reactivation frequencies in brains compared with KOS. In addition, 294.1 induced a low death rate

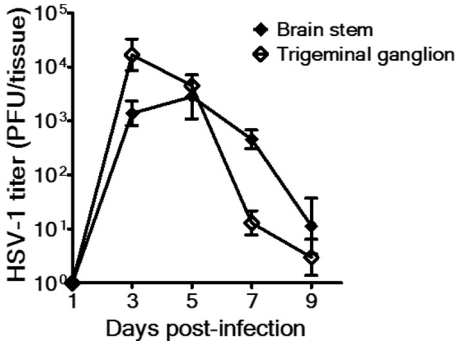

FIG 1 Growth of 294.1 in mouse neural tissues. Brain stems and trigeminal ganglia of mice infected with HSV-1 strain 294.1 were harvested at the indicated times to determine viral titers. The data represent means ± SE of ≥3 samples per data point.

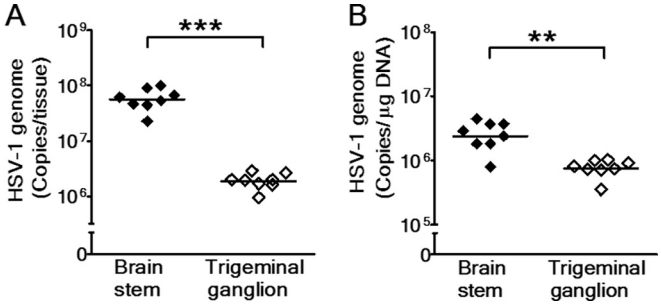

FIG 2 Levels of 294.1 genomes in neural tissues of latently infected mice. Brain stems and trigeminal ganglia of mice infected with 294.1 for 30 days were harvested to quantify the numbers of copies of viral genomes per tissue (A) or per μg of tissue DNA (B). Each point on the scattergrams represents an individual sample, and the horizontal lines represent the mean values for each group. **, $P < 0.01$; ***, $P < 0.001$; Mann-Whitney U test.

in mice compared with McKrae. For mouse strains, C57BL/6N mice were resistant to 294.1- or McKrae-induced lethality compared with ICR and BALB/c mice. Therefore, C57BL/6N mice were inoculated with 1×10^7 to 3×10^7 PFU of 294.1 topically on the right cornea following scarification. After infection, 19% (13/70) of female mice and 40% (6/15) of male mice succumbed to death around 9 to 12 days postinfection (p.i.), so female mice were used for further investigation.

We first monitored the growth of 294.1 in the brain stem and the trigeminal ganglion. Brain stems were chosen for investigation because our previous *ex vivo* study found this brain region had a high HSV-1 reactivation frequency compared with other CNS regions (frontal cortex, cerebellum, olfactory bulbs, hippocampus, and spinal cord) after cultivating dissociated tissue explants (10). Additionally, recurrent brain stem encephalitis has been reported in patients (20). Mouse neural tissues were harvested 1 to 9 days p.i., frozen, homogenized, frozen, sonicated, and subjected to plaque assay for 3 days to determine viral titers. In the mouse trigeminal ganglion and brain stem, 294.1 titers reached peaks on days 3 and 5 p.i., respectively, and then declined (Fig. 1).

On day 30 p.i., mouse brain stems ($n = 8$) and trigeminal ganglia ($n = 8$) were harvested, frozen, homogenized, frozen, sonicated, and subjected to plaque assay for 3 days to detect infectious virus. Infectious virus was not detected in any samples, showing that 294.1 established latency in these neural tissues. We compared HSV-1 latency (DNA) levels in the brain stem and the trigeminal ganglion using quantitative real-time PCR. The average weight of total DNA extracted from one brain stem was about 9-fold higher than that extracted from one trigeminal ganglion (234 ± 12 versus 25 ± 1 μg). The average number of 294.1 genomes in one brain stem was significantly (25-fold) higher than that in one trigeminal ganglion (7.7 ± 0.1 versus 6.3 ± 0.1 log copies) (Fig. 2A) ($P < 0.001$; Mann-Whitney U test). We also calculated the number of 294.1 genomes in 1 μg of tissue DNA and found that the number of viral genomes in the brain stem was also significantly (3.4-fold) higher than that in the trigeminal ganglion (2.7×10^6 versus 7.9×10^5 copies; $P < 0.01$; Mann-Whitney U test) (Fig. 2B).

The brain stem yields reactivated virus earlier than the trigeminal ganglion in mice following hyperthermic stimulation. Treatment of latently infected mice with hyperthermia in a 43°C water bath for 10 min has been used to induce HSV reactivation in the trigeminal ganglion *in vivo* (19, 21–23). We applied this method to compare the brain stem and the trigeminal ganglion for HSV-1 reactivation *in vivo*, so mice latently infected with 294.1 for 30 days were subjected to hyperthermia. Mouse neural tissues were harvested after treatment, frozen, thawed, homogenized, frozen, thawed, sonicated, and plated on Vero cell monolayers to monitor the cytopathic effect, an indicator used in previous studies to detect reactivated (infectious) virus (19, 21, 22, 24). After 6 days, all the cultures were harvested, frozen, thawed, sonicated, and subjected to plaque assay for 4 days to further detect and confirm the presence of infectious virus. We performed reconstitution experiments to assess the sensitivity of this two-step detection assay and found that the assay could detect 10 PFU of HSV-1 in the trigeminal ganglion or brain stem samples.

Using the two-step detection assay, we found that all of the brain stems ($n = 6$) and trigeminal ganglia ($n = 6$) harvested from mice 10 h after hyperthermic stimulation failed to yield infectious virus (Fig. 3A). Brain stems harvested from mice 13, 16, 19, 22, 28, and 34 h after hyperthermic stimulation yielded infectious virus with reactivation frequencies of 26 to 56%. Surprisingly, only the trigeminal ganglia harvested from mice 16 and 28 h after hyperthermic stimulation yielded infectious virus, with reactivation frequencies of 9% (1/11) and 11% (1/9), respectively. In one mouse with reactivated virus detected in the trigeminal ganglion 16 h after hyperthermic stimulation, reactivated virus was not detected in the brain stem. In one mouse with reactivated virus detected in the trigeminal ganglion 28 h after hyperthermic stimulation, reactivated virus was also detected in the brain stem. At 13 h after hyperthermic stimulation, 26% (6/23) of brain stems yielded infectious virus with a frequency significantly higher than that of trigeminal ganglia (0/23; $P < 0.05$; Fisher's exact test). From 13 to 34 h after hyperthermic stimulation, 33% (20/61) of brain stems yielded reactivated virus with a frequency 10-fold higher than that of trigeminal ganglia ($P = 0.02$; Fisher's exact test).

The two-step detection assay was unable to measure the amount of reactivated virus. To quantify the amount of reactivated virus in the same way as in previous reports (23, 25–29), neural tissues harvested from latently infected mice treated with hyperthermia were frozen, homogenized, frozen, sonicated, and subjected to plaque assay. To assess the assay, we performed reconstitution experiments by adding 25 PFU of 294.1 to neural tissues from latently infected mice without hyperthermia treat-

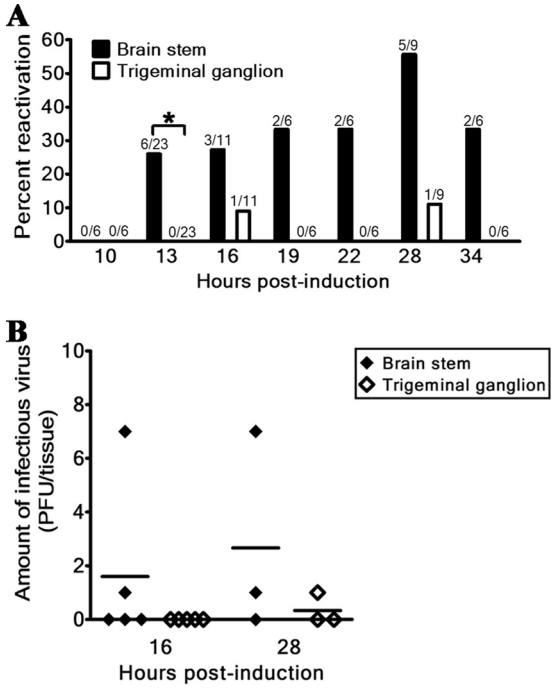

FIG 3 *In vivo* reactivation of 294.1 in mouse neural tissues. Mice infected with 294.1 for 30 days were subjected to hyperthermia to reactivate virus *in vivo*. Mouse brain stems and trigeminal ganglia were harvested at the indicated times after hyperthermia to detect infectious virus (A) or to determine viral titers by plaque assay (B). (A) The numbers above the bars are the numbers of samples positive for reactivated virus out of the number of samples tested. *, $P < 0.05$; Fisher's exact test. (B) Each point on the scattergram represents an individual sample, and the horizontal lines represent the mean values for each group.

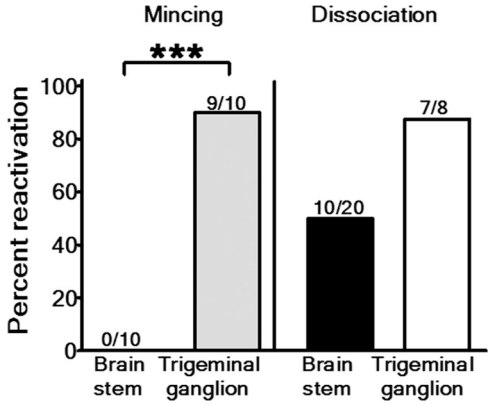

FIG 4 *Ex vivo* reactivation of 294.1 in mouse neural tissues. Brain stems and trigeminal ganglia of mice infected with 294.1 for 30 days were harvested to assay for viral reactivation by the *ex vivo* explant assay using the mincing or dissociation method. The numbers above the bars are the numbers of samples positive for reactivated virus out of the number of samples tested. ***, $P < 0.001$; Fisher's exact test.

ment before processing for the assay. The mean numbers of PFU detected in the trigeminal ganglion and brain stem samples were 8 and 2 PFU, respectively. Using plaque assays for samples harvested at the time points (16 and 28 h after hyperthermic stimulation) when reactivated virus was detected in both the brain stem and trigeminal ganglion, 4 out of 8 brain stems yielded infectious virus, with 1, 1, 7, and 7 PFU of virus per sample, respectively (Fig. 3B), whereas only 1 out of 8 trigeminal ganglia yielded infectious virus, with 1 PFU of virus in the sample. Collectively, the *in vivo* reactivation results revealed that the brain yielded reactivated virus earlier, at a higher frequency, and with more reactivated virus than the trigeminal ganglion.

The *ex vivo* (explant) assay using cultivation of freshly minced or dissociated tissue explants was applied previously to compare HSV reactivation in the mouse brain stem and trigeminal ganglion (10). For comparison with the hyperthermia reactivation model, we also performed the *ex vivo* assay on neural tissues harvested from mice infected with 294.1 for 30 days. After cocultivating minced tissue explants with Vero cell monolayers for 14 days, none of 10 brain stems yielded reactivated virus, whereas 90% (9/10) of trigeminal ganglia yielded reactivated virus (Fig. 4). After cocultivating dissociated tissue explants with Vero cell monolayers for 14 days, 50% (10/20) of the brain stems and 88% (7/8) of the trigeminal ganglia yielded reactivated virus (Fig. 4). The results obtained from *ex vivo* (mincing and dissociation) assays are consistent with previous studies using 294.1 and other HSV-1 strains, which all exhibit low reactivation frequencies in the brain stem compared with the trigeminal ganglion (6, 9–11, 13, 15).

We next compared the *in vivo* reactivation of HSV-1 in the brain stems and the trigeminal ganglia of mice infected with another HSV-1 strain. C57BL/6N mice were infected with 3×10^5 PFU of strain McKrae on the right cornea. This viral dose induced death in 25% (4/16) of female mice and 48% (10/21) of male mice, so female mice were used for further investigation. On day 30 p.i., McKrae established latency in mice, as demonstrated by the failure to detect infectious virus in any of 6 brain stems and 6 trigeminal ganglia by plaque assay. The average number of McKrae latent genomes in one brain stem was significantly (32-fold) higher than that in one trigeminal ganglion ($P < 0.01$; Mann-Whitney U test) (Fig. 5A). In 1 μg of tissue DNA, the average number of McKrae latent genomes in the brain stem was also significantly (4-fold) higher than that in the trigeminal ganglion ($P < 0.01$; Mann-Whitney U test) (Fig. 5B).

Mice infected with McKrae for 30 days were subjected to hyperthermic stimulation, and mouse neural tissues were harvested 10 to 34 h after stimulation to detect infectious virus. Again, all of the brain stems ($n = 6$) and trigeminal ganglia ($n = 6$) harvested from mice 10 h after hyperthermic stimulation failed to yield infectious virus (Fig. 5C). Brain stems harvested from mice 13 to 34 h after hyperthermic stimulation yielded infectious virus. Trigeminal ganglia harvested from mice 16 to 34 h, except at 22 h, after hyperthermic stimulation yielded infectious virus. From 13 to 34 h after hyperthermic stimulation, 14 out of 59 (24%) brain stems and 9 out of 58 (16%) trigeminal ganglia yielded reactivated virus. We also quantified the amount of virus reactivated in mouse neural tissues *in vivo* by plaque assay. Three out of 12 brain stems yielded infectious virus, with 1, 2, and 6 PFU of virus per sample, respectively (Fig. 5D), and 2 out of 12 trigeminal ganglia yielded infectious virus, with 1 and 9 PFU of virus per sample, respectively, 16 and 28 h after hyperthermic stimulation.

DISCUSSION

In the present study, we detected HSV-1 reactivation in the brains earlier than in the trigeminal ganglia of latently infected mice following stimulation. This result proves that the latent HSV-1 ge-

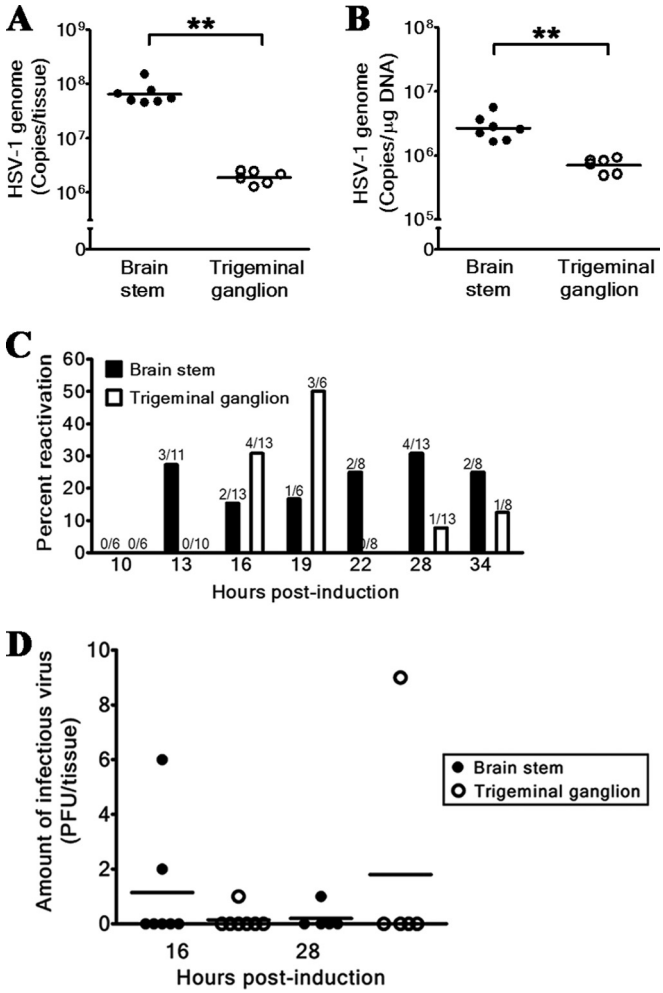

FIG 5 Levels of viral genomes and reactivation in neural tissues of mice latently infected with McKrae. (A and B) C57BL/6N mice were infected with HSV-1 strain McKrae. On day 30 postinfection, mouse brain stems and trigeminal ganglia were harvested to quantify the numbers of copies of viral genomes per tissue (A) or per μg of tissue DNA (B). (C and D) The mice were also subjected to hyperthermia to reactivate virus. Mouse brain stems and trigeminal ganglia were harvested at the indicated times after hyperthermia to detect infectious virus (C) or to determine viral titers by plaque assay (D). (A, B, and D) Each point on scattergrams represents an individual sample, and the horizontal lines represent the mean values for each group. **, $P < 0.01$; Mann-Whitney U test. (C) The numbers above the bars are the numbers of samples positive for reactivated virus out of the number of samples tested.

nomes in the brain are competent to reactivate *in vivo*. More importantly, this result shows that recurrent brain infection can be induced by latent virus reactivating in the brain *in situ*, not necessarily by the spread of reactivated virus from ganglia.

Our *in vivo* results reveal brain stems with high reactivation frequencies (33% and 24% in mice latently infected with 294.1 or McKrae, respectively) compared with trigeminal ganglia (16% and 3% in mice latently infected with McKrae or 294.1, respectively). The *in vivo* results are inconsistent with the *ex vivo* (explant) results, which reveal brain stems with low reactivation frequencies (50% and 0% when assayed by the mincing or dissociation method, respectively) compared with trigeminal ganglia (about 90%). Our previous *ex vivo* study found that brain stem cells lose viability much more rapidly than trigeminal ganglion cells after dissociation and, notably, after mincing (10). This finding may explain the inconsistency between *in vivo* and *ex vivo* results. As the brain and the trigeminal ganglion display differential cell survival after explant, it might not be appropriate to apply the *ex vivo* results to predict viral reactivation in the brain and the ganglion *in vivo*.

Our previous *ex vivo* study using four HSV-1 strains with different degrees of neurovirulence and three mouse strains with variations in their susceptibilities to HSV-1 infection found a strong correlation between the levels of viral genomes and reactivation in the latently infected brain stem (15). Similarly, in the latently infected mouse trigeminal ganglion, previous *in vivo* reports found a positive correlation between the levels of HSV-1 genomes and hyperthermia-induced recurrent infection (22, 30). Here, we demonstrated high levels of viral genomes and hyperthermia-induced recurrent infection in the brain stem compared with the trigeminal ganglion. These studies suggest that the number of copies of latent viral genomes might be an important determinant for the occurrence of recurrent infection in neural tissues *in vivo*.

It has been shown that, in the trigeminal ganglia of latently infected mice treated with hyperthermia, HSV-1 reactivation is restricted to one to a few neurons per event and that the spread of virus in the tissue does not occur in immunocompetent mice (19, 23, 26–28). In the present study, using immunocompetent mice, although hyperthermia induced viral reactivation in the brain stem, the amount of reactivated virus was small (less than 10 PFU per tissue). Mice failed to display signs of encephalitis, such as ataxia and paralysis, after hyperthermic stimulation. Additionally, no obvious lesions and infiltrates were detected in the brain stems of mice latently infected with 294.1 and treated with or without hyperthermia as determined by hematoxylin-eosin staining, indicating that the recurrent brain stem infection may not result in encephalitis. These results showed subclinical reactivation in the mouse brain and also suggest the potential of subclinical reactivation in the brains of immunocompetent humans. In patients, immunodeficiency has been shown to contribute to the development of fatal HSV encephalitis, especially during primary infection (31–33). In mice, immunosuppression has been shown to promote recurrent brain infection *in vivo* (34, 35). We are currently testing whether recurrent brain infection in immunocompromised mice could cause encephalitis.

In patients, recurrent brain stem encephalitis has been reported, but the case number is much less than for encephalitis localized in the frontal and/or temporal cortex (20, 36, 37). Our present study employed mice inoculated with virus by the ocular route, which mimics natural infection in some humans and has been the most frequently used model for investigating HSV reactivation. We previously discovered that after ocular infection, the mouse brain stem had high viral titers, high levels of latent genomes, and a high reactivation rate (assayed by cultivating dissociated tissue explants) compared with the mouse frontal cortex (10). Additionally, 26% and 56% of brain stems, but none of 4 frontal cortices, yielded reactivated virus at 13 and 28 h, respectively, after 294.1-infected mice were treated with hyperthermia. These results collectively revealed that inoculation of mice by the ocular route serves as a good model for investigating recurrent brain stem infection.

Our previous *ex vivo* investigation revealed that HSV-1 strains vary in their reactivation frequencies in the mouse brain stem

(15). In this *in vivo* study, we observed that the differences in early or efficient reactivation of 294.1, but not of McKrae, from the brain stem when compared with the trigeminal ganglion *in vivo* are significant. Additionally, in our model using C57BL/6N mice, the amount of HSV-1 recovered from the trigeminal ganglion (<1 PFU/tissue) was much less than that recovered from Swiss Webster mice (about 10 to 20 PFU/tissue) (25–28). In the future, the issue of whether the early HSV-1 reactivation detected in the mouse brain *in vivo* can be found under other conditions (viral strain, mouse strain, input titer, and inoculation route) needs further investigation.

The longstanding concept that recurrent HSV-1 infection in the brain is induced by the spread of reactivated virus from ganglia has impaired studies investigating viral reactivation in the brain for more than 3 decades. The present *in vivo* study found recurrent brain infection in the absence of reactivated virus in ganglia. This finding broadens our understanding of viral reactivation in the brain, addresses past misconceptions, and, most importantly, provides impetus and a model to initiate studies of this recurrent, devastating disease in the CNS.

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