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# Herpes simplex replication and dissemination is not increased by corticosteroid treatment in a rat model of focal Herpes encephalitis

Katherine A Thompson[1], William W Blessing[2] and Steven L Wesselingh*[,1]

*[1]Infectious Diseases Unit, School of Medicine, Monash University, Victoria 3181, Australia and [2]Department of Medicine, Centre for Neuroscience, Flinders University, South Australia 5042, Australia*

**Neurological damage in Herpes simplex type 1 encephalitis results from neuronal cell death secondary to viral invasion, and from inflammatory changes and cerebral oedema secondary to the immune response to the virus. Corticosteroids could have an important role in the management of Herpes simplex encephalitis because their anti-inflammatory action reduces cerebral oedema. However their use has been limited by concerns that their immunosuppressive actions could increase viral replication and spread. The present study examined this issue in a rat model in which injection of HSV-1 into the cervical vagus nerve produced a well-defined focal encephalitis, characterised by an orderly progression of the virus through central neural pathways connected with vagal afferent termination sites in the medulla oblongata. After injection of HSV-1, rats were treated twice a day, either with vehicle (saline, 400 $\mu$l i.p.), with acyclovir (30 mg/kg i.p.), with dexamethasone (5 mg/kg i.p.), or with both acyclovir and dexamethasone. Animals were sacrificed after 72 h, and viral load in different brain regions was quantified by computer-assisted measurement of the area occupied by immunohistochemical reaction product. Treatment with acyclovir reduced viral load to $17 \pm 5\%$ of the saline value ($P < 0.01$). After dexamethasone treatment, the viral load ($63 \pm 13\%$ of the saline value) was also reduced ($P < 0.05$). Treatment with both acyclovir and dexamethasone reduced viral load to $26 \pm 8\%$ of the saline value ($P < 0.01$ compared with saline, and $P > 0.05$ compared to acyclovir alone). Our results confirm the effectiveness of acyclovir in a new model of HSV-1 infection, and provide evidence that corticosteroids do not inhibit the antiviral action of acyclovir. In addition corticosteroids may decrease the extent of infection in their own right. The acute time course studied in our model parallels the time course of acute Herpes simplex encephalitis in humans. Our data suggests that corticosteroids are not detrimental when combined with acyclovir in the management of this condition.** *Journal of NeuroVirology* (2000) **6,** 25 – 32.

**Keywords:** Herpes simplex virus; encephalitis; acyclovir; corticosteroids; immune modulation

## Introduction

Herpes simplex virus type 1 (HSV-1) causes a sporadic encephalitis with significant morbidity and mortality. Herpes simplex encephalitis (HSE) occurs in approximately 1 in 250 000 to 1 in 500 000 individuals each year (Whitley, 1991; Stroop and Baringer, 1989). Although encephalitis is a rare manifestation of HSV-1 infection, this agent is the most common cause of sporadic (non epidemic) viral encephalitis (Bale, 1993; Roizman and Kaplan, 1992). Acyclovir (9-[(2-hydroxyethoxy)methyl] guanine sodium) and other synthetic purine nucleoside analogues are established as the therapy of choice for the management of HSE (Hayden, 1995; Whitley and Lakeman, 1995; Schaeffer, 1982). The therapeutic use of acyclovir in HSE has been well documented in animal models (Kern *et al*, 1982; Collins and Oliver, 1982; Park *et al*, 1979; Marks, 1975). In clinical

*Correspondence: SL Wesselingh
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trials acyclovir reduced mortality to 19% compared with 50% for virarabine use, and 70% for placebo (Dorsky and Crumpacker, 1987; Campbell *et al*, 1982). However despite optimal medical therapy there is often a prolonged symptomatology and development of long term neurological sequelae (Aurelius, 1993). This has prompted further consideration of adjunctive treatments with immune modulatory agents such as corticosteroids.

HSV-1 usually infects the subfrontal and medial temporal lobes of the brain, where neuropathological examination reveals acute inflammation, congestion and haemorrhage, changes which may proceed to frank necrosis and liquefaction. The cytotoxic action of HSV-1 is associated with an immune response which itself contributes to the inflammatory process. Blood–brain barrier disruption, vasodilation and the action of adhesion molecules results in transudation of intravascular fluid into the brain. The resulting cerebral oedema is a potentially lethal consequence of HSE (Bale, 1993), since rigid confines of the skull and spinal canal leave little room for expansion and displacement before significant compression of neural tissue occurs. Temporal lobe herniation may lead to coma and progressive loss of brain stem function, culminating in respiratory arrest and circulatory failure (Greenlee, 1995; Kalat, 1992).

Corticosteroids are effective agents in controlling inflammation and certain forms of cerebral oedema (Barnes and Adcock, 1993; Brooks *et al*, 1986). Although their use in viral encephalitis has been considered, their role is still controversial because of concern that their immunosuppressive action may result in accelerated viral replication and spread within the nervous system, thereby worsening the final outcome (Fishman, 1982; Kaufman *et al*, 1963). A review of the clinical literature reveals very little useful data to help make decisions regarding the use of corticosteroids in HSE (O'Day, 1991; Upton *et al*, 1971a,b; Habel and Brown, 1971; Longson and Beswick, 1971; Galicich *et al*, 1961). In addition there is a paucity of animal data on the effects of corticosteroids on HSE (Baringer *et al*, 1976; Dobrzynski, 1970).

The present study utilises a new animal model of Herpes encephalitis, one which has been extensively characterised in studies of the transneuronal spread of HSV-1. In these studies the orderly progression of viral antigen through delineated CNS pathways has been well documented (Blessing *et al*, 1991, 1994). After being inoculated into one cervical vagus nerve, the virus is transported into the vagal afferent cell bodies in the nodose ganglion. After replication, the virus reaches the medulla oblongata via the central processes of the vagal afferents. The virus initiates a focal encephalitis in the ipsilateral medulla, and then spreads by retrograde axonal transport to the parent cell bodies of brain neurons with axons projecting directly to the medullary sites of afferent vagal termination. Within a 72 h survival period, sites of spread include the rostral portion of the contralateral ventrolateral medulla oblongata and the ipsilateral central nucleus of the amygdala. The virus also spreads to glial cells closely related to infected neurons, including the afferent vagal rootlets which pass through the ventrolateral medulla oblongata on the side ipsilateral to the injected cervical vagus.

The vagal injection model lends itself to quantitative measurement of infected brain tissue, thus facilitating a study of the effect of corticosteroids on the viral load in the brain. We used this new animal model of Herpes encephalitis to determine whether or not corticosteroids, by themselves or in combination with acyclovir, are associated with any increase in the area of the nervous system infected by the virus.

## Results

*Distribution of HSV-1 antigen in the brain*

HSV-1 antigen was detected in the medulla oblongata ipsilateral to the side of vagal injection in every rat, regardless of the treatment administered. The immunohistochemical staining was dense in the nucleus tractus solitarius and in the spinal tract and nucleus of the trigeminal nerve, sites of termination of vagal afferents. Staining was also observed along the intramedullary course of the vagal afferent rootlets, with spread to surrounding glial cells. In rats treated with intraperitoneal saline the ipsilateral medullary labelling was quite extensive as described in Blessing *et al* (1991) and also demonstrated in Figure 1. In the contralateral medulla oblongata staining was intense in the nucleus tractus solitarius (the vagal projection is bilateral with ipsilateral predominance) and in the ventrolateral medulla where there is a concentration of nerve cells with direct projections to the nucleus tractus solitarius. In saline treated rats there was usually dense staining in the ipsilateral central nucleus of the amygdala, with lighter staining on the contralateral side (Figure 2). Labelling was also seen elsewhere in the brain as described in Blessing *et al* (1991) but for the purposes of the present study we quantified the staining in the selected regions.

*Viral load in ipsilateral medulla oblongata*

After intraperitoneal saline treatment, the area of HSV-1 immunohistochemical staining in the ipsilateral medulla (average of caudal and rostral sections) was $2.6 \pm 0.4$ mm$^2$. As can be seen in Figure 3a, treatment with acyclovir substantially reduced the immunohistochemically stained area to $0.9 \pm 0.1$ mm$^2$ ($P < 0.01$). After dexamethasone treatment, the area of HSV-1 immunohistochemical staining was $1.9 \pm 0.4$ mm$^2$ ($P > 0.05$ compared with saline). Treatment with both acyclovir and dex-

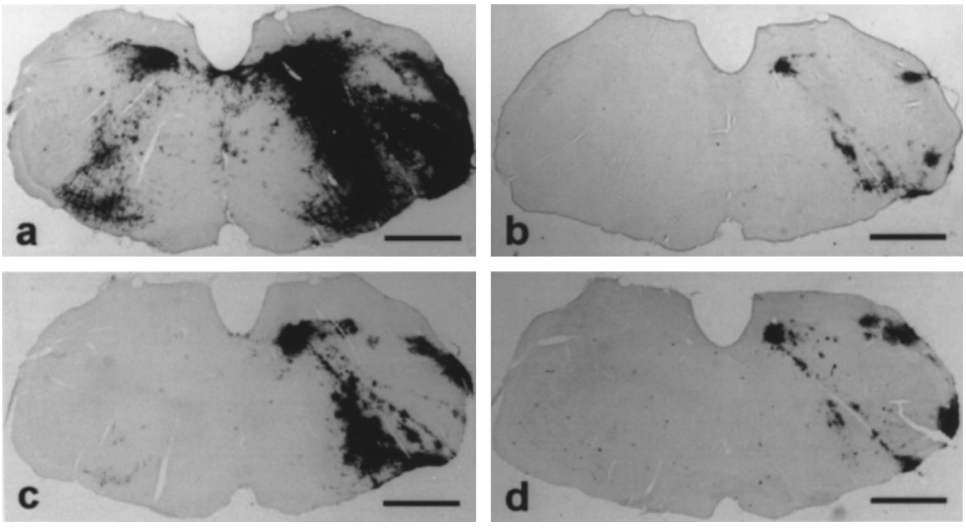


**Figure 1** Photomicrographs of rostral ventolateral medulla transverse sections of 4 week-old rats, 3 days after injection of HSV-1 into the left cervical vagus nerve, immunohistochemically stained for HSV-1 antigens. Saline treatment (**a**), acyclovir treatment (**b**), dexamethasone treatment (**c**), and acyclovir and dexamethasone treatment (**d**). Bar=1 mm (**a–d**).

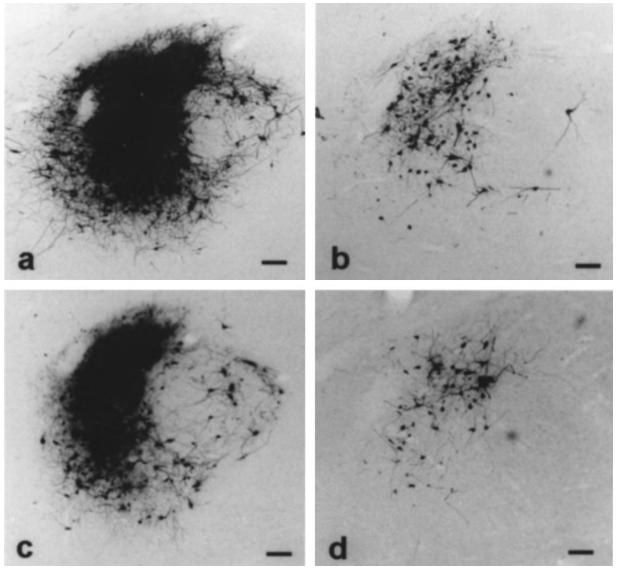


**Figure 2** Photomicrographs of transverse ipsilateral amygdala cross-sections of 4 week-old rats, 3 days after injection of HSV-1 into the left cervical vagus nerve, immunohistochemically stained for HSV-1 antigens. Saline treatment (**a**), acyclovir treatment (**b**), dexamethasone treatment (**c**), and acyclovir and dexamethasone treatment (**d**). Bar=100 $\mu$m (**a–d**).

amethasone also significantly reduced the affected area to $1.1 \pm 0.4$ mm$^2$ ($P<0.05$ compared with saline). The area of staining after acyclovir treatment alone was not significantly different from the area of staining after combined acyclovir and dexamethasone treatment ($P>0.05$).

*Viral load in contralateral rostral ventrolateral medulla oblongata*

After intraperitoneal saline treatment, the area of HSV-1 immunohistochemical staining in the contralateral rostral ventrolateral medulla was $0.3 \pm 0.1$ mm$^2$. As can be seen in Figure 3b, treatment with acyclovir substantially reduced the immunohistochemically stained area to $0.02 \pm 0.02$ mm$^2$ ($P<0.01$). After dexamethasone treatment, the area of HSV-1 immunohistochemical staining was $0.14 \pm 0.08$ mm$^2$ ($P>0.05$ compared with saline). Treatment with both acyclovir and dexamethasone also significantly reduced the affected area to $0.04 \pm 0.02$ mm$^2$ ($P<0.01$ compared with saline). The area of staining after acyclovir treatment alone was not significantly different from the area of staining after combined acyclovir and dexamethasone treatment ($P>0.05$).

*Viral load in ipsilateral amygdala*

After intraperitoneal saline treatment, the area of HSV-1 immunohistochemical staining in the ipsilateral amygdala was $0.4 \pm 0.1$ mm$^2$. As can be seen in Figure 3c, treatment with acyclovir substantially reduced the immunohistochemically stained area to $0.01 \pm 0.01$ mm$^2$ ($P<0.01$). After dexamethasone treatment, the area of HSV-1 immunohistochemical staining was $0.2 \pm 0.1$ mm$^2$ ($P>0.05$ compared with saline). Treatment with both acyclovir and dexamethasone also significantly reduced the affected area to $0.1 \pm 0.04$ mm$^2$ ($P<0.01$ compared with saline). The area of staining after acyclovir alone treatment was not significantly different from the area of staining after combined acyclovir and dexamethasone treatment ($P>0.05$).

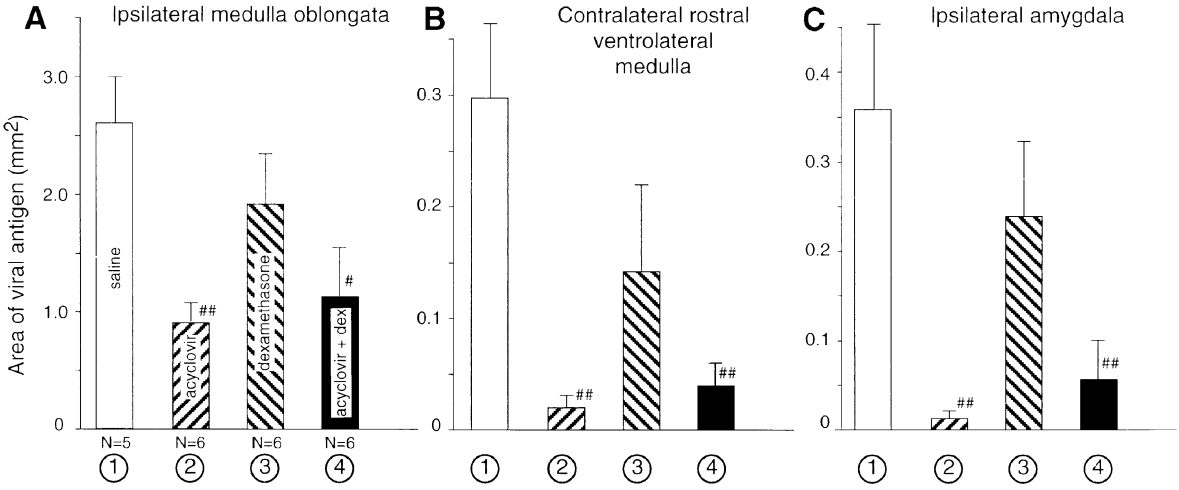


**Figure 3** Area of HSV-1 antigen ($mm^2 \pm$s.e.m.) in ipsilateral medulla oblongata (**a**), contralateral rostral ventrolateral medulla (**b**), and ipsilateral amygdala (**c**) of 4 week-old rats 3 days post HSV-1 injection into the left cervical vagus nerve. Saline treatment (1), acyclovir treatment (2), dexamethasone treatment (3), and acyclovir and dexamethasone treatment (4). ##Significantly less than saline treatment, $P<0.01$. #Significantly less than saline treatment, $P<0.05$.

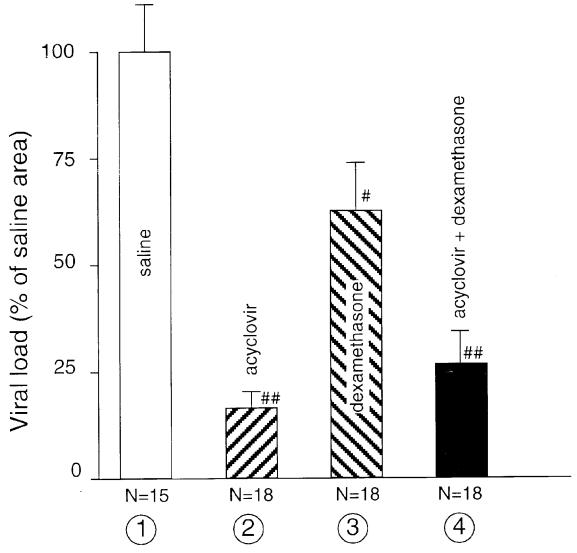


**Figure 4** Area of HSV-1 antigen expressed as a percentage of saline area (%$\pm$s.e.m.), in combined areas of measurement from 4 week-old rats 3 days post injection of HSV-1 into left cervical vagus nerve. Saline treatment (1), acyclovir treatment (2), dexamethasone treatment (3), and acyclovir and dexamethasone treatment (4). ##Significantly less than saline treatment, $P<0.01$. #Significantly less than saline treatment, $P<0.05$.

*Overall percentage measure of viral load in the brain*

The three brain regions measured (ipsilateral medulla, contralateral rostral ventrolateral medulla, ipsilateral amygdala) were combined to obtain an overall measure of brain viral load by expressing the mean stained area of HSV-1 antigen as a percentage of saline area.

After intraperitoneal saline treatment, the area of HSV-1 immunohistochemical staining in the combined areas of measurement was $100 \pm 12\%$. As can be seen in Figure 4, treatment with acyclovir reduced the immunohistochemically stained area to $17 \pm 5\%$ ($P<0.01$). After dexamethasone treatment, the area of HSV-1 immunohistochemical staining ($63 \pm 13\%$) was also significantly reduced compared to saline ($P<0.05$). Treatment with both acyclovir and dexamethasone reduced the affected area to $26 \pm 8\%$ ($P<0.01$ compared with saline). The percentage area of staining after acyclovir alone was not significantly different from the area of staining after combined acyclovir and dexamethasone treatment ($P>0.05$).

## Discussion

Acyclovir treatment substantially reduced the HSV-1 viral load in the region of primary brain infection (ipsilateral medulla oblongata) and in the two regions (contralateral rostral ventrolateral medulla and ipsilateral amygdala) selected for measurement of viral spread within the central nervous system. Acyclovir treatment reduced the overall brain viral load area measurement to 16% of the infected area in saline treated rats. This demonstration of the effectiveness of acyclovir confirms its use in the treatment of Herpes simplex encephalitis.

Our model makes it possible to specifically measure effects of antivirals on viral replication and spread *in vivo*. Previous models of HSE treated

with acyclovir have been extensively reported (Narang, 1995; Schinnazi *et al*, 1986; Kern *et al*, 1982; Collins and Oliver, 1982; Park *et al*, 1979). The initiation of HSE in these models is primarily through intracerebral injection which produces widespread infection over multiple regions of the brain, making specific measurements such as viral spread difficult. Therefore the majority of outcome measurements are non specific and include percentage mortality and mean day of death. In contrast, our new model makes it possible to determine the effect of antivirals on viral replication and spread. Death is not the only outcome in HSE. Even with acyclovir treatment, 19% of patients are left with long term neurological sequelae (Stroop and Baringer, 1989). The obvious advantage of the rat model presented here is that specific measures of viral replication and spread can be assessed and statistically analysed.

Addition of dexamethasone to acyclovir produced a very small non-significant increase in viral load in the three regions of measurement compared with acyclovir treatment alone. Thus the immunosuppressive action of dexamethasone did not result in increased viral replication and spread within the nervous system. Furthermore there was no increase in infectivity in any of the brain regions examined when dexamethasone was administered alone, without acyclovir. Indeed, in the overall combined percentage analysis of viral load in the brain, dexamethasone, by itself, actually reduced the infected area to 63% of the value in saline treated rats.

Baringer *et al* (1976) reported similar findings. Effects of the corticosteroid, prednisolone, were evaluated using a rabbit model of HSE in which HSV-1 was inoculated on the scarified cornea. Corticosteroid treatment showed no significant difference in viral titre measurement when compared with infected control animals. The rate of clearance of virus from the brain stem appeared slightly slower in the corticosteroid treated group. However virus titres were reduced to zero levels in all animals, despite continued corticosteroid treatment. Comparison of the histologic features in the prednisolone treated *versus* the control animals showed no difference in the time of appearance or size of lesions, the degree of necrosis, or appearance of haemorrhage.

There are a number of possibilities as to how dexamethasone may reduce viral replication or spread. One possibility is via an effect on glia. In transneuronal tracing studies performed by Blessing *et al* (1994), glia appeared to be infected earlier than neurons. Blessing *et al* (1994) suggested that the virus may pass from the axon terminal of the peripheral neuron to brain glial cells before it enters other neurons. Immunostimulated proliferation of these cells could provide increased host pools for viral production thus increasing viral infection. Dexamethasone treatment may decrease cell proliferation inhibiting viral replication and spread.

Alternatively dexamethasone could have direct effects on viral replication or spread. Infected cells die from necrosis or through programmed cell death, i.e. apoptosis (Kerr and Hamon, 1991). Apoptosis, induced in the virally infected host cells, could prevent viral replication, thereby preventing further viral spread. It is possible that dexamethasone could induce virally infected astrocytes to undergo more rapid apoptosis, preventing complete viral replication and production of new viral particles, also preventing further spread.

This study confirms the effectiveness of acyclovir in a new highly reproducible model of HSV-1 infection in which both viral load and viral spread can be quantitatively measured. It also provides evidence that corticosteroids do not inhibit the antiviral action of acyclovir, and that corticosteroids may decrease the extent of infection in their own right. Our data therefore suggest that corticosteroids are more likely to be beneficial rather than harmful in treatment of Herpes simplex encephalitis, and should be considered in the appropriate clinical setting.

## Materials and methods

*Preparation of HSV-1 viral stocks*

A clinical isolate of HSV-1 was obtained and grown under sterile conditions in Hep2 cells in DMEM, supplemented with 10% foetal calf serum, penicillin and streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$. HSV-1 titres were determined by an indirect immunofluorescent assay. Fifty microlitre aliquots of virus stock were stored at −70°C.

*HSV-1 injection procedure*

Female Fisher rats, approximately 4 weeks old (100–150 g) were anaesthetised with sodium pentobarbitone (60 mg/kg i.p.). Approximately 1 cm of the left vagus nerve was exposed, approximately 2 cm below the nodose ganglion. HSV-1 preparation ($10^5$ p.f.u. per $\mu$l of innoculum) was then thawed and made easily visible by the addition of a small amount of Pontamine blue (BDH Chemicals, UK). The blue solution was drawn up into a glass micropipette via polyethylene tubing connected to an air-filled 10 ml syringe. The field was viewed through an operating microscope. The vagal epineurium was grasped and holding the micropipette almost parallel to the nerve, the tip was inserted into the nerve for approximately 2 mm. Approximately 1 $\mu$l of innoculum was injected into the nerve which could be seen to turn blue in its course towards the nodose ganglion. The wound was sutured and the animal was kept warm while it recovered from the anaesthetic.

*Administration of treatments*
HSV-1 injected rats were divided into four groups (six rats in each group), with each group receiving a different treatment. Each treatment was administered intraperitoneally twice per day for the 72 h survival period, starting on the morning following the injection. Group 1 received sodium chloride vehicle (400 $\mu$L i.p.). Group 2 received acyclovir (30 mg/kg i.p.). Group 3 received dexamethasone (5 mg/kg i.p.). Group 4 received both acyclovir and dexamethasone (doses as for Groups 2 and 3). Dosages were based on those previously reported for acyclovir treatment in HSE animal models (Narang, 1995; Field *et al*, 1995).

*Tissue fixation and sectioning*
After 72 h, each rat was reanaesthetised (sodium pentobarbitone 180 mg/kg i.p.) and perfused transcardially with 4% formaldehyde/15% picric acid in 0.1 M sodium phosphate buffer pH 7.4, followed by 15 and 30% sucrose in the same solution. The brain was then removed and immersed in fixative solution (4% formaldehyde/15% picric acid in 0.1 M PBS, pH 7.4+20% sucrose) overnight at 4°C. The brain was then placed in 0.5 M PBS, pH 7.4, containing 30% sucrose at 4°C for 1–2 days. A freezing microtome was used to cut serial 40 $\mu$m transverse sections.

*Immunohistochemical detection of viral antigen*
Sections were washed four times for 15 min in 50% ethanol and left overnight in 0.1 M tris buffered saline (TBS), pH 7.4. Sections were incubated in 20% normal horse serum (NHS) in 0.1 M TBS pH 7.4, at room temperature for 1 h. The sections were rinsed in 1% NHS/TBS then a polyclonal rabbit anti-HSV-1 (Dakopatts, Denmark) diluted 1 : 5000 in 1% NHS/TBS was added and sections were incubated overnight at 4°C. Sections were then washed three times for 10 min in 1% NHS/TBS, then incubated in biotinylated goat anti-rabbit IgG diluted 1 : 200 in 1% NHS/TBS overnight at 4°C. Sections were washed three times for 10 min in 1% NHS/TBS and incubated with an avidin-biotin-peroxidase complex (1 : 50) for 1 h at room temperature. Sections were washed in 0.1 M TBS pH 7.4, for 10 min, then incubated in 0.05% diaminobenzidine for 10 min at room temperature. Hydrogen peroxide was added to a final concentration of 0.01% and the colour reaction allowed to proceed for 5–10 min depending on degree of staining. The reaction was stopped by washing the sections in 0.1 M TBS. Sections were mounted on slides, dehydrated through graded ethanol solutions and cleared in Xylene, and coverslipped.

*Quantification of viral load in medulla and central nucleus of the amygdala*
Sections were examined using an Olympus BH-2 microscope and photographed at low magnification with a digital camera (Canon EOS.DC5) connected to the microscope. Images were transferred to an Apple Macintosh Quadra 840 AV computer using Adobe Photoshop 3.0.5. An eye graticule in the microscope was used to measure the section size, and the digital photomicrograph was calibrated according to this measure. Immunohistochemically demonstrated HSV-1 antigen was present in neurons and in glial cells. The staining was very dark and confluent in different brain regions so that it was often visible with the naked eye, as previously published (Blessing *et al*, 1991).

Viral load was quantified by measuring the area of confluent immunohistochemical reaction product in a given tissue section, taken from a specified level of the medulla oblongata or through the region of the forebrain containing the central nucleus of the amygdala. Immunohistochemical detection of viral antigen expression was utilised as a measure of productive infection and hence viral load. It is possible that HSV-1 DNA in some cells does not result in viral antigen expression. However this would account for only a very small number of cells and not significantly alter the outcome measurements. Additionally, since the activity of acyclovir is to reduce productive viral replication, cells not expressing viral antigens would therefore not influence the results. For each rat, two medullary sections were chosen from defined rostrocaudal levels of the medulla; a caudal section from the mid area postrema level, and a rostral section from the level where the nucleus tractus solitarius is positioned ventral to the vestibular nuclei and the nucleus ambiguus is prominent (level of the rostral ventrolateral medulla). We used image software (National Institute of Health, USA) to measure areas which contained viral antigen, as determined by appropriate thresholding of the image so that areas containing the dark immunohistochemical reaction product were counted as positive. For each rat we averaged the results from the two medullary levels to give a single viral antigen area for the ipsilateral medulla. On the contralateral side, only the stained region within the rostral ventrolateral medulla was included in the area measurement. Viral antigen reaches this region only by retrograde intraaxonal transport to neurons which project to the site of primary afferent vagal termination in the nucleus tractus solitarius. In the forebrain, we measured the area of viral staining in the ipsilateral amygdala at the rostrocaudal level corresponding to the greatest development of the central nucleus of the amygdala. Finally, in each rat, we obtained an overall measure of brain viral load by expressing the mean stained area in each of the three brain regions (ipsilateral medulla, contralateral rostral ventrolateral medulla, ipsilateral amygdala) for saline treated animals as 100%, and then calculating the percentage area score for each rat in a particular treatment condition for each of the three brain regions. Data were then

combined, resulting in 15 percentage measures for the saline treated rats, 18 percentage measures for acyclovir treatment alone, 18 for dexamethasone alone and 18 for both drugs administered together.

All measurements were made without knowledge of the treatment group to which a particular rat belonged. Area measurements ($mm^2$) were expressed as mean $\pm$ standard error of mean (s.e.m.). Analysis of variance (with Fishers Least Significant Difference *post-hoc* comparison) was used to determine differences between the four treatment groups in each of the three different brain regions, and in the overall percentage measure of viral load. Differences were considered significant if $P < 0.05$.

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