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Neuronal activity regulates viral replication of herpes simplex virus type 1 in the nervous system

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Herpes simplex virus types 1 and 2 (HSV-1, -2) infect and also establish latency in neurons. In the present study, the authors investigated the influence of neuronal activity on the replication of HSV-1. The results showed that the sodium channel blocker tetrodotoxin (TTX) and the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) could significantly increase viral replication in primary neuronal cultures, by two- to fourfold. In contrast, KCl reduced viral production by at least 80% in the same cultures. Inhibitors of $GABA_A$ receptors completely abolished the effects of GABA. Intravitreously injected TTX in a mouse corneal scarification model enhanced the viral titers >10-fold in both the trigeminal ganglia and the brain. At 2 h post infection, both TTX and GABA significantly up-regulated the levels of transcription for the viral immediate early (IE) genes ICP0, ICP4, and ICP27, as revealed by real time PCR. These results indicate that the neuronal excitation status may dictate the efficiency of HSV-1 viral replication, probably by regulating the levels of viral IE gene expression. These are the first findings connecting neuronal activity to the molecular mechanisms of HSV replication in the nervous system, which may significantly influence our view of herpesvirus infection and latency. *Journal of NeuroVirology* (2005) **11,** 256–264.

Keywords: GABA; herpes simplex virus; immediate early gene; neurons; TTX; viral replication

Introduction

Herpes simplex virus (HSV) types 1 and 2 are common viruses causing various pathological conditions in humans (Knopf, 2000; Roizman and Sears, 1990; Taylor *et al*, 2002a). HSV virus can remain latent in the infected neurons for life and can be reactivated under certain circumstances (Cohrs and Gilden, 2001; Millhouse and Wigdahl, 2000a; Rajcani and Durmanova, 2000a). Encephalitis is a common component of disseminated HSV infection and about 60% to 70% of newborns that contract encephalitis are herpetic (Schmutzhard, 2001). In addition, HSV is the most common cause of sporadic fatal encephalitis in adults. Herpes infection is one of the most common complications in acquired immunodeficiency syndrome (AIDS) patients (Cavert, 1997; Itin and Lautenschlager, 1997; Krzyzowska *et al*, 2000; Reichart, 1997; Sparling *et al*, 2001; Wald, 2002). Recently, it has been suggested that HSV infection may also contribute to Down syndrome and Alzheimer's disease (Cheon *et al*, 2001; Dobson and Itzhaki, 1999; Itzhaki, 2004; Mori *et al*, 2004).

Replication of HSV-1 begins with the transcription of five immediate early (IE) viral genes (ICP0, ICP4, ICP22, ICP27, and ICP47) (Knopf, 2000; Millhouse and Wigdahl, 2000a). These in turn activate the early and late viral genes. Among IE genes, ICP4 and ICP27 are absolutely essential for viral replication (Weir, 2001). In most cases, HSV-1 establishes latency in neuronal cells of sensory ganglia (Mitchell *et al*, 2003). The latently infected virus can be reactivated throughout the life of the host under various conditions, such as trauma (Gordon *et al*, 1990; Huang *et al*, 1999; Kameyama *et al*, 1989; McGill and Cartotto, 2000), cold (Blondeau *et al*, 1993; Everett, 2000b), heat stress (Hunsperger and Wilcox, 2003;

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Loiacono *et al*, 2003), ultraviolet (UV) irradiation (Laycock *et al*, 1991; Loiacono *et al*, 2003), adrenergic iontophoresis (Nesburn *et al*, 1983; Willey *et al*, 1984), and immunosuppression (Higaki *et al*, 2002; Marquart *et al*, 2003). Although it has been known that ICP0, ICP4, and ICP27 genes are essential for viral reactivation from latency, the detailed molecular mechanism remains unclear (Everett, 2000b; Millhouse and Wigdahl, 2000b; Mitchell *et al*, 2003; Rajcani and Durmanova, 2000b; Taylor *et al*, 2002b). More recently, transgenic mice carrying a reporter gene regulated by various HSV-1 viral gene promoters have been generated (Loiacono *et al*, 2002; Loiacono *et al*, 2003, 2004). One interesting possibility suggested from the results of those studies is that ICP0 and ICP27 genes may be induced in neurons without requiring the presence of viral proteins (Loiacono *et al*, 2002). Thus, expression of those genes that are necessary for viral reactivation may be regulated by the cellular proteins in neurons.

When rat sensory neurons from dorsal root ganglia (Mayer *et al*, 1985, 1986; Mayer, 1986) were infected with HSV-1, spontaneous action potential activity was induced in these cells. Furthermore, simultaneous recording from pairs of infected neurons indicated that the virus had induced electrical coupling between sensory neurons, presumably as a result of syncytial formation. Morphological and electrophysiological studies on cultured neurons from the dorsal root ganglia of guinea pigs infected with herpes simplex virus *in vivo* have indicated that electrophysiological techniques are much more sensitive than immmnofluorescence methods for the detection of HSV infection in nerve cells (Fukuda and Kurata, 1981; Fukuda *et al*, 1983). Measurement of membrane excitability after HSV infection in tissue-cultured rat dorsal root ganglia nerve cells (Fukuda and Kurata, 1981) showed that a loss of excitability occurs much earlier than any other change in the HSV-infected nerve cells. This loss of excitability was due to a reduction in neuronal sodium conductance, and was attributed to a virally induced modification of host cell protein synthesis (Oakes *et al*, 1981). More recent works showed that different viral strains have different effects on the excitability of infected sensory neurons in rats (Mayer, 1986) and that virally triggered changes in excitability were blocked by acyclovir, suggesting that viral adsorption and penetration are by themselves insufficient to evoke changes in excitability. Therefore, the effects of herpes viruses on excitable mechanisms in sensory neurons are not simply the result of a general loss of membrane conductance or a disruption of transmembrane ion gradients. Neuronal excitability is ultimately determined by the activity of ion channels on the cytoplasmic membrane. Sodium, potassium, and calcium ion influx depolarizes the cell and raises the excitability whereas chloride ion influx hyperpolarizes and inhibits cell excitation. The activity of ion channels on neuronal cells is regulated by neurotransmitters through specific receptors or drugs acting on specific ion channels. Excitatory neurotransmitters such as glutamate activates neurons by opening calcium channels and causes depolarization of the membrane. On the other hand, inhibitory neurotransmitters, such as gamma-aminobutyric acid (GABA), hyperpolarize the membrane by opening the chloride channel (Calver *et al*, 2002; Frolund *et al*, 2002; Johnston, 2002; Sieghart and Sperk, 2002); (Haines and Ard, 2002), which suppresses the excitability of the cell. Neuronal excitability can also be regulated by many drugs, such tetrodotoxin (TTX) and KCl. TTX inhibits the neuronal activity by selectively suppressing the voltage-dependent Na^+ current and abolishes the action potential on the neuronal cytoplasmic membrane (Strichartz *et al*, 1987). KCl excites neurons by depolarizing the membrane directly (Kandel *et al*, 2000).

Our previous study showed that the neurovirulence of HSV-1 in animals infected through the corneal-trigeminal ganglion route was much more severe than that in animals infected directly in the cortex (Song and Jia, 1999). A similar observation was also made in the mouse nervous system (Mitchell, 1995; Wharton *et al*, 1995). These observations indicated that viral replication in the neurons depends on cell type and location in the nervous system, which determine the pattern of neuronal cell activity. Thus, we hypothesized that the neuronal activity may regulate the viral activity. To establish if this is the case, in the present study, we manipulated the excitability of neuronal cells through regulating ion channels on the cell surface, and investigated the influence on HSV viral gene expression and replication in infected neurons.

Results

Neuronal activity regulates HSV-1 viral production in primary neuronal cultures

After the neurons had been infected with HSV-1 at multiplicity of infection (MOI) = 0.1 and the cells had been incubated with various drugs that altered neuronal excitability through ion channels, the virus generated in the infected neurons was titered by plaque assays on Vero cells. As shown in Figure 1a, KCl inhibited HSV-1 replication in the neurons by 80% (19.8% ± 3.0% of the control, $P < .001$, $n = 6$) at 60 mM, a concentration commonly used to depolarize neuronal cells.

Suppression of spontaneous neuronal activity was achieved by applying TTX or GABA to the cultures used in the present study. Treatment with TTX and GABA resulted in increased viral production. The titers of neuronal cultures treated with TTX and GABA were 415% ± 68.7% ($P < .001$, $n = 6$) and 247% ± 32.8% ($P < .001$, $n = 6$) those of the controls, respectively (Figure 1b, c). Figure 2 shows the time

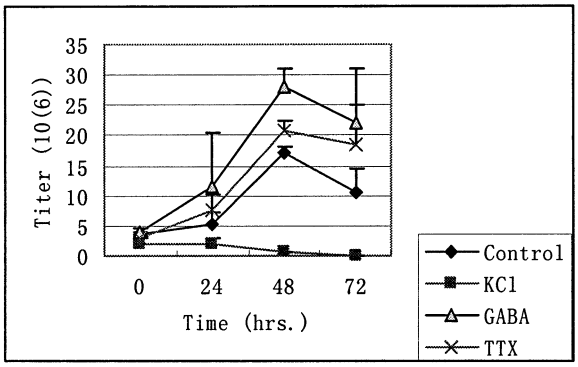


Figure 1 Effects of neuronal ion channel modulators on HSV-1 replication in cultured cortical neurons. Viral titers were measured on Vero cells as the number of PFUs (1×10^6 as indicated in the figure) for 1×10^6 infected neurons. Data are averages from at least two independent experiments each with triple repeats.

course of virus production in neuronal cells with or without the above treatments. It was apparent that the greatest production occurred in TTX (1.5 μM)- or GABA (120 μM)-treated neuron cultures in the first 48 h post infection ($P < .05$). On the other hand, viral production was completely inhibited in KCl (50 mM)-treated neurons. By 72 h, the viral titers had dropped, which may have been caused by neuronal death in the late stage of infection.

To confirm that the effect of GABA on viral replication in neuronal cells was mediated by specific receptors, antagonists for the corresponding receptors were added together with GABA. Two $GABA_A$ receptor antagonists, picrotoxin (40 μM) and bicuculline methiodide (MBI; 120 μM), both completely restored viral titers to the control level in the presence of GABA (120 μM) (Figure 3). Therefore, we presumed that the GABA-caused up-regulation of viral activity was mediated by stimulating the $GABA_A$ receptor.

To verify whether the changes in viral titers were due to an alteration in the characteristics of the cellular membrane affecting viral entry into the cells, we treated neurons with the above agents before or after viral inoculation. No difference in titers was found (data not shown) between the two groups, indicating that the altered neuronal activity affected viral replication at postentry stages. To rule out the possibility that the drug treatment may have altered the plaque-forming ability of viral particles on Vero cells, we further measured total viral DNA (Figure 4a) and viral proteins (Figure 4b) from infected neurons directly using Southern blotting on the viral IE gene ICP4 and Western blotting on the total viral proteins with a polyclonal HSV-1 antibody. Consistent with the results of plaque assays, KCl (50 mM) reduced the amounts of total viral DNA and proteins significantly, whereas the inhibitory agents TTX (1.5 μM) and GABA (120 μM) caused increased synthesis of viral DNA and proteins.

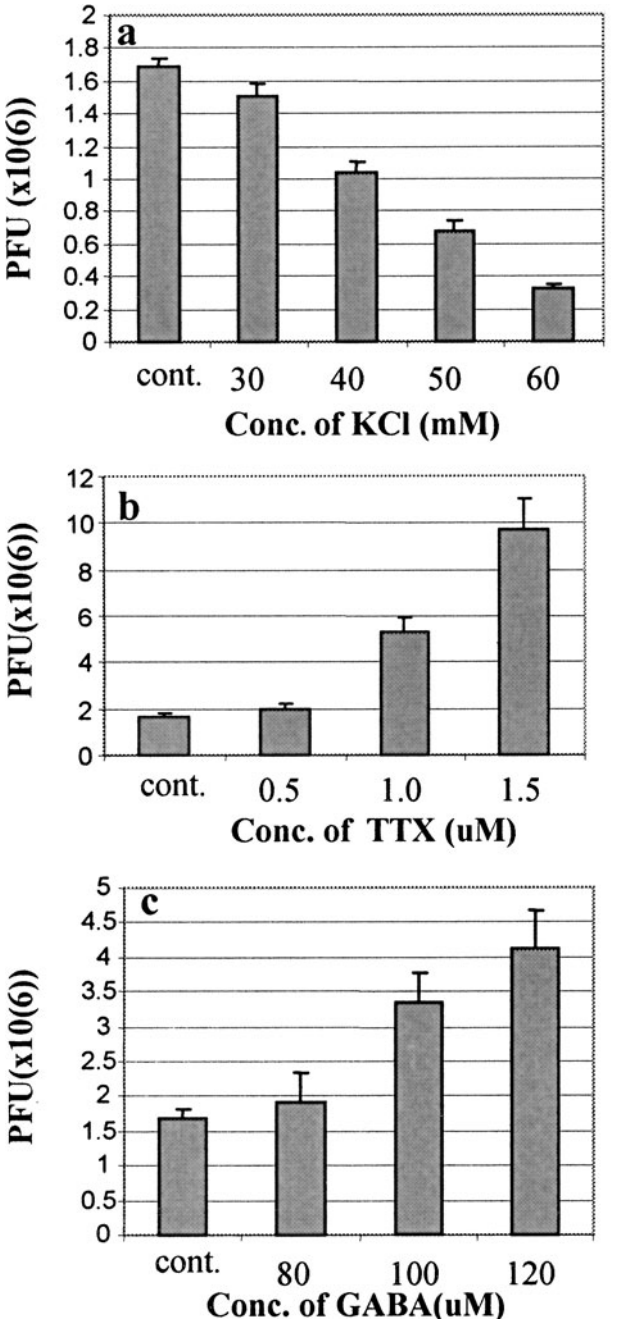
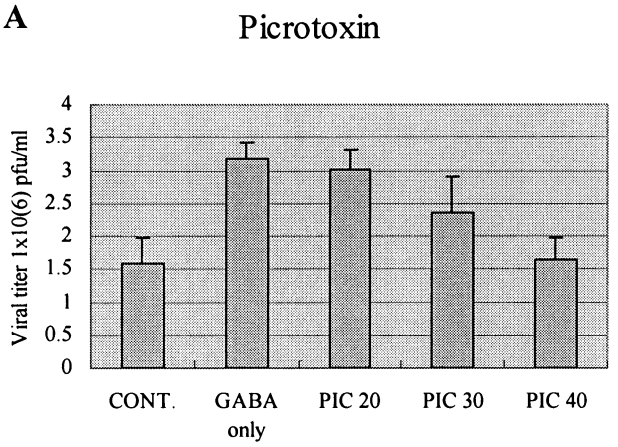


Figure 2 One-step growth of virus production in neuronal cells treated with various neuronal activity modulators. Neuronal cultures were infected with HSV-1 virus at MOI = 8 in the presence of TTX (1.5 μM), KCl (50 mM), or GABA (120 μM). The virus was harvested from the infected cell cultures at various time points and then titered on Vero cells. Data are averages of three repeated experiments each performed in duplicate.

Reduced neuronal activity by TTX facilitated viral replication in a cornea scarification animal model

Viral replication in the nervous system was measured in animals infected with HSV-1 through cornea scarification immediately following an intravitreous injection of either TTX or phosphate-buffered saline



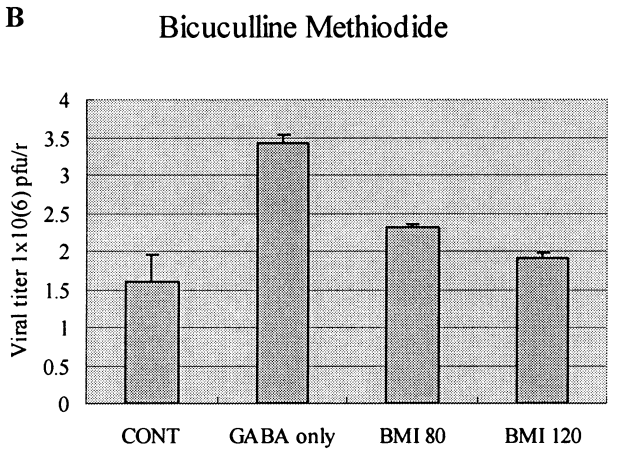


Figure 3 Effects of $GABA_A$ receptor antagonists on virus replication enhanced by GABA in cultured cortical neurons. Two $GABA_A$ receptor antagonists, picrotoxin and bicuculline methiodide (MBI), both blocked the GABA-caused increase in viral replication.

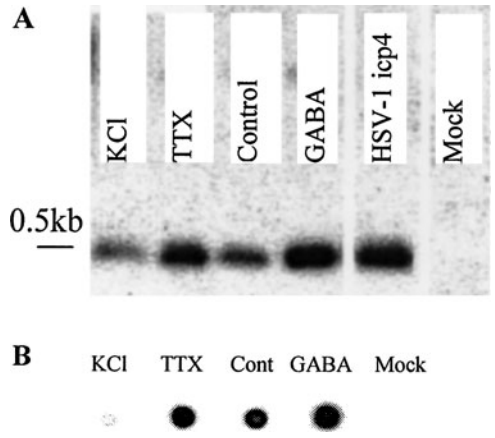


Figure 4 **a**, Southern blotting of the SacI-digested HSV-1 genome using a 487-bp SacI fragment of the ICP4 gene probe labeled with digoxigenin. A plasmid containing HSV-1 ICP4 cDNA was used as the positive control. **b**, Western dot-blotting of total HSV-1 proteins with a polyclonal rabbit anti-HSV-1 antibody (1:1000; DAKO A/S, Denmark). Cont, control sample.

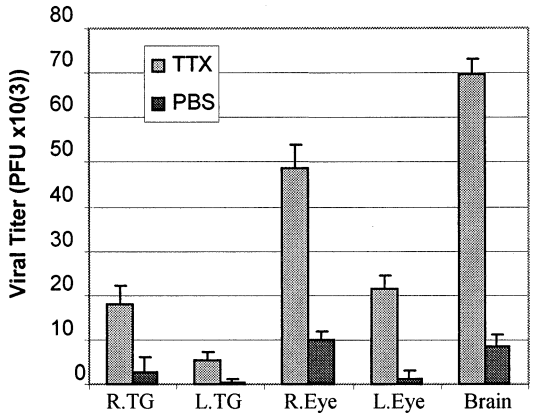


Figure 5 Viral titers in tissues from the cornea-inoculated animals treated with intravitreously injected TTX (3 μl of 1 mM TTX) or PBS control. R.TG and L.TG, right and left trigeminal ganglia, R.eye and L.eye, right and left eye. The viral titers were determined by plaque assays on Vero cells (details in Materials and Methods). The data represent averages of five animals in both TTX and PBS groups.

(PBS). As shown in Figure 5, on the inoculated side (right), the effect of TTX was rather dramatic with 13- and 18-fold increases in the viral titers in the eye and the trigeminal ganglion, respectively (both $P < .001$). Even on the uninoculated side, TTX also significantly facilitated viral replication in the eye and trigeminal ganglion by 6.9- and 4.9-fold, respectively (both $P < .001$). Viral replication was also significantly enhanced by 8.3-fold ($P < .001$) in the brains of TTX-treated animals.

Neuronal activity-regulated efficiency in viral replication is mediated by altering expression of viral IE genes

HSV-1 replication is initiated by expression of IE genes, which are transcriptional factors responsible for the expression of other viral genes. In particular, IE genes ICP0, ICP4, and ICP27 play essential roles in viral replication. We have measured the mRNA levels of these three genes at 2 h post infection with or without the above agents using real-time reverse transcriptase–polymerase chain reaction (qRT-PCR) (Figure 6). Relative to the control that infected with HSV-1 without TTX or GABA, ICP0, ICP4, and ICP27 transcript levels were 249% + 43%, 303% + 28%, and 251% + 32%, respectively, in TTX (1.5 μM)-treated cells and 173% + 20%, 179% + 13%, and 180% + 15%, respectively for GABA (120 μM)-treated neuronal cultures ($P < .001$).

Discussion

The present study investigated the effects on HSV-1 viral replication of neuronal cell activity through the regulation of various ion channels in infected neurons, both in primary cultures and in an animal model. Our results showed that increasing neuronal excitability using KCl could inhibit viral replication

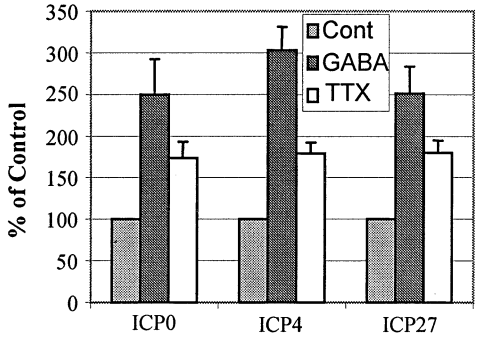


Figure 6 Transcriptional levels of HSV-1 immediate-early genes ICP0, ICP4, and ICP27 at 2 h post infection (MOI = 3) were measured in neurons treated with TTX (1.5 μM) or GABA (120 μM) with real time PCR. The data are expressed as percentages of control samples that were infected with HSV-1 without TTX or GABA treatment. The data shown are averages from at least two repeated experiments.

and decreasing the activity of neurons using sodium channel blockers, such as TTX or with an inhibitory transmitter, GABA, enhanced viral production in neuronal cells and in the nervous system. We further demonstrated that the alteration in ion channel activity in neuronal cells may directly affect the expression of viral immediate early genes, which may subsequently regulate viral replication.

Suppression of spontaneous neuronal activity was also achieved by applying GABA and TTX to the cultures employed in the present study. Activation of $GABA_A$ receptors usually hyperpolarizes neurons by opening anion channels and allowing an influx of chloride ions (Dichter, 1980; McBain and Fisahn, 2001; Moore, 1993). TTX, on the other hand, inhibits the neuronal activity through a completely different mechanism that selectively suppresses the voltage-dependent Na^+ current and abolishes the action potential in neurons (Strichartz *et al*, 1987). This effect was more striking *in vivo,* in which intravitreously injected TTX markedly increased viral titers in the eye, the trigeminal ganglia, and the brain. TTX does not affect other cellular activities of neurons such as fast axonplasmic transport (Nakamura *et al*, 1965; Hille, 1968; Pestronk *et al*, 1976; Butler *et al*, 1978), which is responsible for the retrograde transportation of HSV-1 virions in the nervous system (Tomishima *et al*, 2001). Thus, it is unlikely that TTX increased viral titers in the ganglion and the brain by facilitating viral transportation in axons. The increased titers in the brain were more likely due to TTX-increased viral production in the trigeminal ganglion, which subsequently provided more virion input to various brain regions through retrograde transportation. In addition, because TTX blocked the afferent neuronal activities in the eye, including retinal and trigeminal input, activity in related areas of the brain would be reduced, which may also contribute to enhanced viral replication.

Although the effects of these agents, especially GABA, seem less dramatic on changes in viral replication in primary neuronal cultures than those of other antiviral drugs, they could be quite significant. First, these agents affected viral replication in a clear dose-response fashion, indicating that the seemingly small differences reflected actual effects. Secondly, primary cortical cultures contain heterogeneous populations of neurons and not every neuron expresses $GABA_A$ receptors (Koller *et al*, 1990a, 1990b; Seifert *et al*,; Stichel and Muller, 1991). This would explain why the response to GABA was less than that to TTX, which may have silenced all the neurons in the culture and caused a nearly fivefold increase in HSV-1 replication. Thirdly, neither GABA nor TTX directly inhibit the virus because applying them on Vero cells had no effect on viral replication (data not shown). Instead, these agents alter the activity of host cells to create a favorable environment for viral replication and provide an important model for understanding how neuronal activity influences the efficiency of viral replication. Finally, a few fold change in viral replication may have a significant effect on the nervous system in vivo. In our mouse cornea scarification model, the total virus titers at the peak of replication in the eyes, ganglia and brain were only in the range of 10^4 plaque-forming units (PFU), which was much less than we normally see in cell culture systems. In addition, some recent studies have shown that only 3% of neurons harvest HSV-1 in human trigeminal ganglia (Cai *et al*, 2002) and there are probably only 20 to 200 copies of the viral genome in each neuron (Chen *et al*, 2002; Cohrs *et al*, 2000; Thompson and Sawtell, 2000). Again, this very small amount of viral particles is sufficient to cause reactivation of infection and even encephalitis. Thus, a few-fold increase in efficiency of viral replication *in vivo* may be enough to completely change the status of viral activity in the ganglia or brain.

It has been known for a long time that ionphresis of adrenergic agents can reactivate latently infected HSV-1 in the cornea (Gordon *et al*, 1986; Harwick *et al*, 1987; Kwon *et al*, 1981; Shimomura *et al*, 1983). Interestingly, it is also known that adrenergic neurotransmitters suppress neuronal excitability in neurons of the trigeminal ganglia (Cahusac *et al*, 1995). This is consistent with our findings that inhibition of neuronal activity by GABA or TTX enhanced viral replication.

Although all the above agents can modulate neuronal activity through regulating ion channels on the cell surface, it is not known whether their influence on HSV-1 replication at this stage makes use of the same mechanism. HSV-1 viral replication has several stages starting with viral entry, IE gene expression, viral-DNA synthesis, and viral-particle packaging and egress. Because no difference in viral titers was found in neurons treated with the above agents either before or after viral inoculation (data not shown), the stage of viral entry may not be affected. It is more likely that these neural activity modulators interfere with viral replication at the second stage, i.e., the

expression of IE genes. This is supported by the observation that levels of IE gene transcripts were elevated at a very early stage after infection in the presence of TTX and GABA. ICP4 and ICP27 are transcriptional factors that are responsible for the initial expression of many viral early genes. Although ICP0 is not essential, its deletion results in significantly reduced viral replication (Everett, 2000a). In addition, all of the IE proteins are required for reactivation of latent HSV-1 virus in the central nervous system (CNS) (Rajcani and Durmanova, 2000a; Roizman, 1999). Based on our findings, it is reasonable to speculate that neuronal activity regulates levels of the viral IE gene expression that dictate the efficiency of viral replication and reactivation from latency, although the underlying molecular mechanisms remain to be revealed.

To our knowledge, this is the first study that correlates the excitability of neuronal cells with HSV-1 viral replication in neurons. It is particularly interesting that neuronal ion channels regulated by GABA, the major inhibitory transmitter in the nervous system, can significantly influence viral IE protein levels through specific receptors at an early stage of viral infection. Thus, GABA activity may contribute to the replication, latency, and reactivation of HSV-1 in the nervous system. Understanding the effect of neuronal activity on viral replication may potentially lead to important new approaches to the treatment of a variety of neurological diseases caused by primary and recurrent HSV infection.

Materials and methods

Cells and virus
Primary neuronal cultures from rat embryonic cerebral cortex (17 to 18 days of gestation) were prepared and grown in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) following the protocol described by Dichter (1978). On day 3, the medium was replaced with fresh medium containing the antimitotic drugs FudR and uridine (2×10^{-5} M; both from Sigma) to reduce the nonneuronal cell population (Godfrey *et al*, 1975). Neuronal cells were used after 14 to 16 days in culture to ensure the maturation of ion channels (Koller *et al*, 1990a, 1990b; Seifert *et al*, 1983; Stichel and Muller, 1991). Vero cells (African Green Monkey Kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and 1% antibiotics.

The HSV-1, KOS strain was used throughout the present study. The virus was produced in Vero cells and harvested by centrifugation at 5000 $\times$ *g* at 4°C for 10 min. The supernatant was further centrifuged at 10,000 $\times$ *g* at 4°C for 2 h to pellet the virions. Virion pellets were resuspended in PBS and respun through a 20% sucrose gradient for 2 h at 10,000 $\times$ *g* at 4°C. Pellets were then resuspended with PBS and stored in aliquots at −70°C.

Drugs and chemicals
TTX, KCl, GABA, and the $GABA_A$ receptor antagonists BMI and picrotoxin (PIC) were all purchased from Sigma Chemical.

Viral infection and drug treatments
Fourteen-day-old primary neuronal cultures were infected with HSV-1 virus at a MOI of 0.1 for 45 min at 37°C, in an atmosphere of 5% CO_2. The cultures were washed twice with MEM and incubated in MEM + 10% FBS medium containing drugs at different doses for 24 h at 37°C. In preliminary experiments, we tested the possible toxicity of these drugs on neuronal cultures by measuring the cell viability with both MTT and lactate dehydrogenase (LDH) activity (Pauwels *et al*, 1990) assays. Toxicities of TTX, KCl, GABA, BMI, and PIC at various concentrations were tested on neuronal cultures and Vero cells with both MTT and LDH assays. None of the drugs in the concentrations used had any cytotoxic effect (data not shown).

Virus plaque assay
The medium of the infected neuronal cultures was removed and the culture dishes were frozen and thawed three times. Following centrifugation, the supernatants were used to infect near confluent Vero cell monolayers in DMEM + 5% FBS medium for 1 h, then the medium was replaced with fresh medium containing 0.01% human immunoglobulin G (IgG) and incubated at 37°C for 48 h. The cells were fixed with 4% paraformaldehyde and stained with methylene blue. The number of plaques was counted under a dissecting microscope.

Southern blotting
A 487-bp SacI fragment isolated from a previously cloned 6.3-kb BamHI fragment containing HSV-1 ICP4 cDNA was labeled with digoxigenin using a Dig DNA labeling kit (Roche Diagnostics). Total DNA from the infected neuronal cells was isolated and purified with a DNAzol reagent (GIBCO BRL) and digested with SacI. The DNA (6 μg DNA/lane) was separated on a 1% agarose gel and transferred to a nylon membrane. Following hybridization with the labeled 487-bp SacI fragment, the ICP4 gene was detected by chemiluminescence, using the manufacturer's protocol (P/E Life Sciences)

Intravitreous injection and corneal HSV-1 infection
Two groups of Balb/C mice consisting of five mice each were anesthetized and injected with 3 μl of 1 mM TTX or PBS (for control animals) into the anterior chamber of the right eye with a microsyringe. Immediately after the injection, the cornea of the same eye was scratched 10 times with a 27-gauge needle, followed by application of 5×10^3 PFU KOS virus in a volume of 5 μl. TTX or PBS was injected intravitreously every second day for 6 days. TTX is

known for its long lasting, irreversible effect (Lu *et al*, 2001), which persists for more than 48 h when applied intraocularly (Archer *et al*, 1982; Dubin *et al*, 1986). Animals were sacrificed on day 7 and the eyes, trigeminal ganglia, and brain were collected. Following freezing and thawing, the tissues were homogenized and resuspended in PBS. After centrifugation, the supernatants were collected and stored at −80°C. For viral titration, 1/10 of total volume from the above supernatants was taken to infect Vero cells with a series of dilutions. Plaques formed on Vero cell cultures were counted to determine viral titers for each samples.

Real time PCR

Total RNA was extracted from infected neuronal cells using Trizol Reagent (Invitrogen) and reverse transcription was then carried out using MultiScribe Reverse Transcriptase and random hexamers (both from Applied Biosystems). Real time PCR on HSV-1 ICP0, ICP4, and ICP27 was performed with SYBR Green PCR Mater Mix on a 7000 Sequence Detection System (Applied Biosystems). The mRNA of the β-actin gene was also measured as the internal control. Primers utilized for the transcripts were

ICP0	Forward:	5′-TTACGTGAACAAGACTATCACGGG-3′
	Reverse:	5′-TCCATGTCCAGGATGGGC-3′
ICP4	Forward:	5′-GGCCTGCTTCCGGATCTC-3′
	Reverse:	5′-GGTGATGAAGGAGCTGCTGTT-3′
ICP27	Forward:	5′-GTCTGGCGGACATTAAGGACA-3′
	Reverse:	5′- TGGCCAGAATGACAAACACG-3′
Actin	Forward:	5′-ACGAGGCCCAGAGCAAAGAG-3′
	Reverse:	5′-TCTCCATGTCGTCCCAGTTG 3′

Statistics

An unpaired Student's *t* test was used for statistical analysis with Excel (Microsoft Office 2000) or SPSS.

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