

# Spread of bovine herpesvirus type 5 (BHV-5) in the rabbit brain after intranasal inoculation

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Following intranasal inoculation of wild-type BHV-5 in rabbits, we studied the sequential transneuronal passage of the virus in the CNS by immunocytochemistry, histopathology, and virus isolation. At 4 and 6 days postinfection (d.p.i.), rabbits had no or mild neurological signs, and virus was isolated only from the olfactory bulbs. At 8 and 9 d.p.i., infected rabbits had severe neurological signs, and virus could be isolated from multiple regions of the brain segments. In these rabbits, high titers of virus were consistently present in the anterior and posterior cortices, including frontal, piriform/entorhinal, temporal, parietal, and occipital cortices, the hippocampus and the amygdala. Virus was isolated occasionally from the midbrain/diencephalon and pons/medulla. Virus was not isolated from the cerebellum and trigeminal ganglion of rabbits examined from 2–12 d.p.i. Immunocytochemistry revealed virus-specific antigens at 4 d.p.i. within the glomerular layer, external plexiform layer, and mitral cell layer of the main olfactory bulb. At 6 d.p.i., virus-specific antigens were also present within the inner granular layer of the main olfactory bulb. At 8 and 9 d.p.i., widespread BHV-5-specific staining occurred in the areas of the brain connected to the main olfactory bulb, including the frontal/cingulate cortex, anterior olfactory nucleus, lateral olfactory tubercle, piriform/entorhinal cortex, hippocampus, amygdala, dorsal raphe, and locus coeruleus. In the trigeminal ganglion, specific staining was detected within a few neurons at 2, 4, 6, 8 d.p.i. However, further spread of the virus along the trigeminal pathway was not evident. These data indicate that BHV-5 replicates and spreads preferentially in the olfactory pathway following intranasal instillation and that this viral spread correlated with the severity of neurological symptoms and histopathological lesions.

**Keywords:** BHV-5; neuropathogenesis; CNS-invasion; neurovirulence

## Introduction

Bovine herpesvirus type 5 (BHV-5) is a neurovirulent alphaherpesvirus (d'Offay *et al*, 1993). Experimental intranasal inoculation with BHV-5 results in severe neurological signs in young calves between 9–10 days postinoculation (d.p.i.) (Belknap *et al*, 1994). The virus initially replicates in the olfactory and/or nasal epithelium and subsequently spreads to the central nervous system (CNS) to cause a severe, often fatal, encephalitis in calves (Belknap *et al*, 1994). Nonneurovirulent BHV-1 and neurovirulent BHV-5 infections can be distinguished

based on their differential neuropathogenesis in a rabbit seizure model (Chowdhury *et al*, 1997). In this model, intranasal inoculation of rabbits with BHV-5 causes acute neurological signs that are comparable to those seen in calves. In the brains of infected rabbits, neuronal damage is located preferentially within the hippocampus formation and the piriform/entorhinal and frontal cortices. Virus-specific antigens and nucleic acid are detectable in these affected regions of the brain (Chowdhury *et al*, 1997).

The nasal and olfactory epithelia are innervated by three different types of neurons: (1) Olfactory receptor neurons, located in the olfactory epithelium, are the first order bipolar neurons. They have dendrites directly in contact with external medium and their axons project to several groups of neurons,

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primarily in the main olfactory bulb (Switzer *et al*, 1985); (2) parasympathetic motor neurons in the pterygopalatine (or sphenopalatine) ganglia have axonal connections with the superior salivatory nucleus in the medulla oblongata (Contreras *et al*, 1980; Spencer *et al*, 1990); (3) primary sensory neurons in the trigeminal ganglion have connections with neurons of the pontine and spinal trigeminal nuclei (Sp5) in the pons and medulla oblongata, respectively (Tracey, 1985).

Herpes simplex virus (HSV), pseudorabies virus (PRV), and rabies virus enter the CNS via both the olfactory and trigeminal nerves after intranasal inoculation (Barbic *et al*, 1994; Kritas *et al*, 1994a,b; Lafay *et al*, 1991; Martin and Dolivo, 1983; Stroop *et al*, 1984; Tomlinson and Esiri, 1983). Additionally, HSV-1 and PRV can enter the CNS via the ophthalmic branch of the trigeminal nerve after corneal inoculation (Knotts *et al*, 1974; Martin and Dolivo, 1983; Stroop *et al*, 1984). Following intranasal instillation of vesicular stomatitis virus (VSV) in mice, invasion of the CNS occurred via the olfactory pathway but not the trigeminal pathway (Lundh *et al*, 1987).

Our previous studies suggested that the olfactory pathway is more susceptible than the trigeminal pathway to the spread of BHV-5 after intranasal inoculation of rabbits (Chowdhury *et al*, 1997). In those experiments, rabbits were euthanized after they showed severe neurological signs, usually 8–10 days after virus inoculation. Thus, the systematic progression of BHV-5 infection in the CNS after the virus inoculation was not observed. The purpose of the present study was to map the systemic spread of BHV-5 in the rabbit nervous system after intranasal inoculation. For this purpose, the penetration, spread, and propagation of BHV-5 within the CNS were evaluated at different postinoculation times by immunocytochemistry, histopathology, and virus isolation.

## Results

### *Clinical signs of disease*

The two rabbits sacrificed at 2 d.p.i. did not have any clinical signs. Rabbits sacrificed at 4 and 6 d.p.i. had mild neurological signs characterized by hyperaesthesia, head-twitching and/or trembling. At 8 d.p.i., four rabbits were sacrificed that had severe neurological signs, which consisted of seizures/convulsion, upright sitting posture, circular movement, and opisthotonus. The seizures occurred at 5–10-min intervals and lasted for 1–2 min. At 9 d.p.i., one rabbit had seizures and was sacrificed, and one was found dead (had mild neurological signs until 8 d.p.i.). The remaining four rabbits had only mild neurologic signs and no seizures; two of them were sacrificed at 10 d.p.i. and the other two at 12 d.p.i. (Tables 1 and 2).

### *Virus isolation*

The results of virus isolation from nasal swabs and different brain regions are presented in Table 1. At 2 and 4 d.p.i., BHV-5 was isolated from nasal swabs (Table 1). At 6 d.p.i. and thereafter, no virus could be isolated from nasal swabs.

In the brain, BHV-5 was isolated only from the olfactory bulb of rabbit #2 ( $7.6 \times 10^2$  plaque forming unit (PFU)/g) and rabbit #6 ( $3.4 \times 10^2$  PFU/g) at 4 and 6 d.p.i., respectively. At 8 d.p.i., rabbit #7 had high titers of virus in the olfactory bulb ( $8.0 \times 10^2$  PFU/g), anterior ( $6.0 \times 10^2$  PFU/g) and posterior ( $5 \times 10^3$  PFU/g) cortices, and the midbrain/diencephalon ( $1.8 \times 10^2$  PFU/g). Virus also was isolated from the pons and medulla, but only after repassage of the infected cells. Rabbit #13 had virus in the anterior ( $2 \times 10^1$  PFU/g) and posterior ( $2.4 \times 10^3$  PFU/g) cortices and in the pons/medulla ( $2 \times 10^1$  PFU/g). In rabbit #8, which was found dead at 9 d.p.i., virus was isolated only from the anterior ( $5.4 \times 10^2$  PFU/g) and posterior ( $1 \times 10^4$  PFU/g) cortices.

**Table 1** Virus isolation from nasal swabs and brain tissues

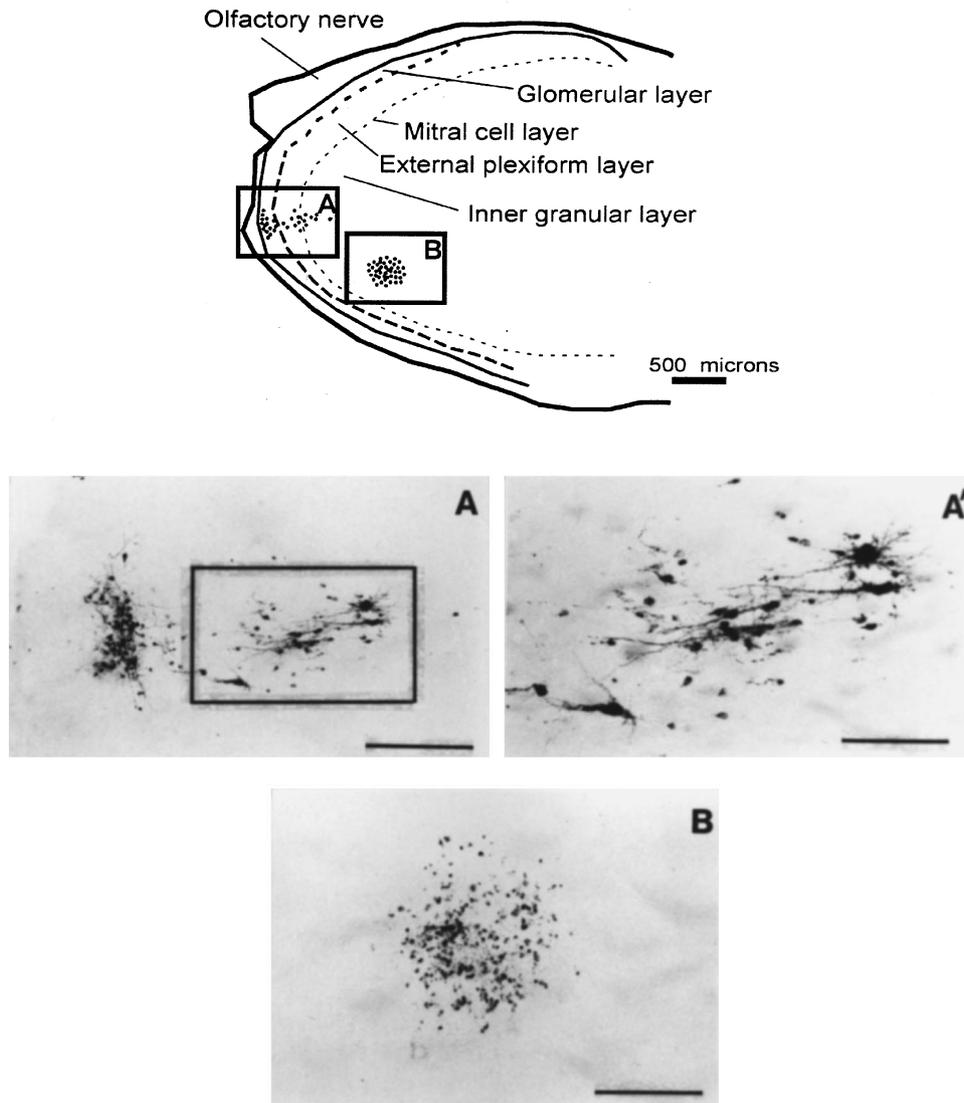
Rabbit #	Neurological signs	Day died/killed (d.p.i.)	PFU's in nasal swab	Localization of virus in the brain						
				Olfactory bulb	Anterior <sup>a</sup> cortex	Posterior <sup>b</sup> cortex	Midbrain/Diencephalon	Pons/Medulla	Cerebellum	Trigeminal ganglion
2	no	2	45	–	–	–	–	–	–	–
4	mild	4	7	++	–	–	–	–	–	–
6	mild	6	–	++	–	–	–	–	–	–
7	seizure	8	–	++	+++	+++	+	+	–	–
13	seizure	8	–	–	+	+++	–	+	–	–
8	found dead	9	–	–	++	+++	–	–	–	–
11	mild	10	–	–	–	–	–	–	–	–
14	mild	12	–	–	–	–	–	–	–	–

no= no clinical signs observed; mild signs are exemplified by hyperaesthesia, head twitching and/or trembling; <sup>a</sup>includes olfactory tract, frontal cortex/cingulate cortex, and the anterior portion of piriform cortex; <sup>b</sup>includes temporal, piriform parietal, occipital, and entorhinal cortices, amygdala and hippocampus; – = no virus detected after repassage; + =  $10^1$ – $10^2$  PFU/g of tissue; ++ =  $10^2$ – $10^3$  PFU/g; +++ =  $10^3$ – $10^4$  PFU/g. +\* = CPE noted after repassage of sample.

**Table 2** Presence and location of BHV-5 virus antigen within the rabbit brain following intranasal inoculation

Rabbit #	Neurological sign	Days killed (d.p.i.)	Localization of virus in the brain										
			OB	Pir	Hippo	Amyg	CG	LC	DR	LDT	Cere	TG	
1	no	2	—	—	—	—	—	—	—	—	—	—	+*
3	mild	4	++	—	—	—	—	—	—	—	—	—	+*
5	mild	6	++	—	—	—	—	—	—	—	—	—	+
10	seizure	8	++	++++	++++	+++	+++	+	++	+	—	—	+*
15	seizure	8	++	++++	++++	+++	+++	+	++	—	—	—	+*
12	seizure	9	++	++++	++++	+++	+++	+	++	—	—	—	—
16	mild	10	—	—	—	—	—	—	—	—	—	—	—
9	mild	12	—	+	—	+	—	—	—	—	—	—	—

— = no labeling, + = 1–25 labeling cells, \* = less than half of the grading range; ++ = 25–150 labeling cells, +++ = 150–500 labeling cells, ++++ = >500 labeling cells per field at 5× magnification. Abbreviations: d.p.i., days postinfection; OB, olfactory bulb; Pir, piriform cortex; Hippo, hippocampus; Amyg, amygdala; CG, cingulate cortex; LC, locus coeruleus; DR, dorsal raphe; LDT, lateral dorsal tegmentum; Cere, cerebellum; TG, trigeminal ganglion.



**Figure 1** Localization of BHV-5 specific antigen in the main olfactory bulb at 4–6 d.p.i. Line drawing of the olfactory bulb (sectioned in sagittal plane). Frames indicate areas shown in photographs. Note that the immunostained neurons were indicated by dots. Immunostained cells had dense black precipitate over the cell nucleus. (A) At 4 d.p.i., immunostained neurons in the glomerular layer, the external plexiform, mitral cell layer and inner granular neurons. (A') Detail of boxed area under higher power magnification. (B) At 6 d.p.i., immunostained neurons in the inner granular neurons. Scale bars: 200 μm (A and B), 100 μm (A').

Virus was not isolated from any sections from the rabbits with mild neurological signs sacrificed at 10 and 12 d.p.i. Virus isolation results were negative from the trigeminal ganglion or cerebellum of all the rabbits, at all time points.

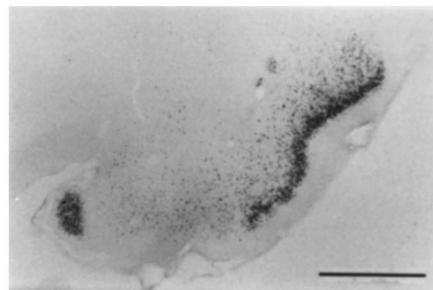
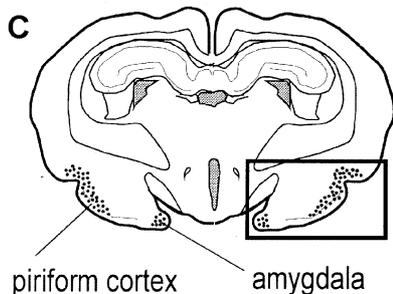
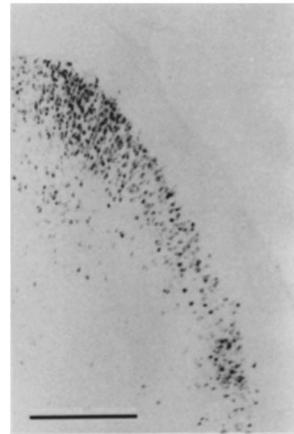
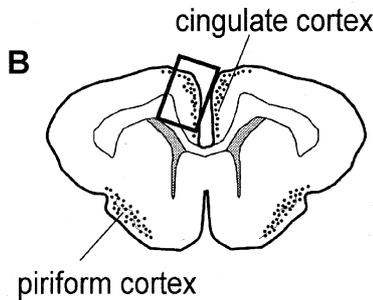
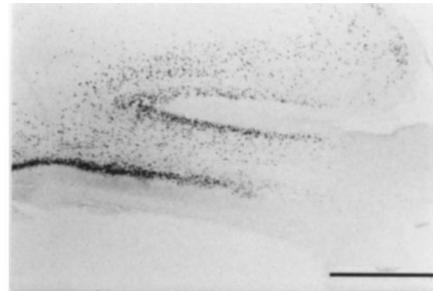
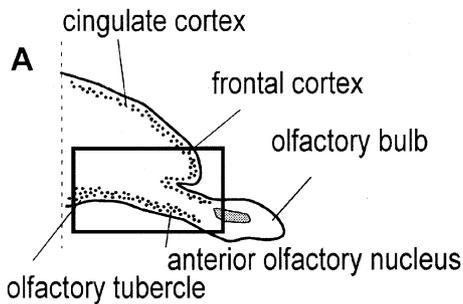
*Histopathological features*

In sections obtained at 2–6 d.p.i., no significant histological lesions were present in any of the regions of the brain that were examined. At 8 d.p.i., scattered neuronal necrosis and lymphocytic perivascular infiltrates occurred infrequently in the olfactory bulb. These lesions were most prominent and numerous in the caudal portions of the bulb. The piriform/entorhinal and frontal/cingulate cortices and hippocampus had locally extensive regions of moderate to severe lymphocytic meningo-encephalitis and perivascular cuffing in associa-

tion with extensive zones of neuronal necrosis, gliosis, and rarefaction of the neuropil (data not shown).

*Time course and antigen localization by immunocytochemistry*

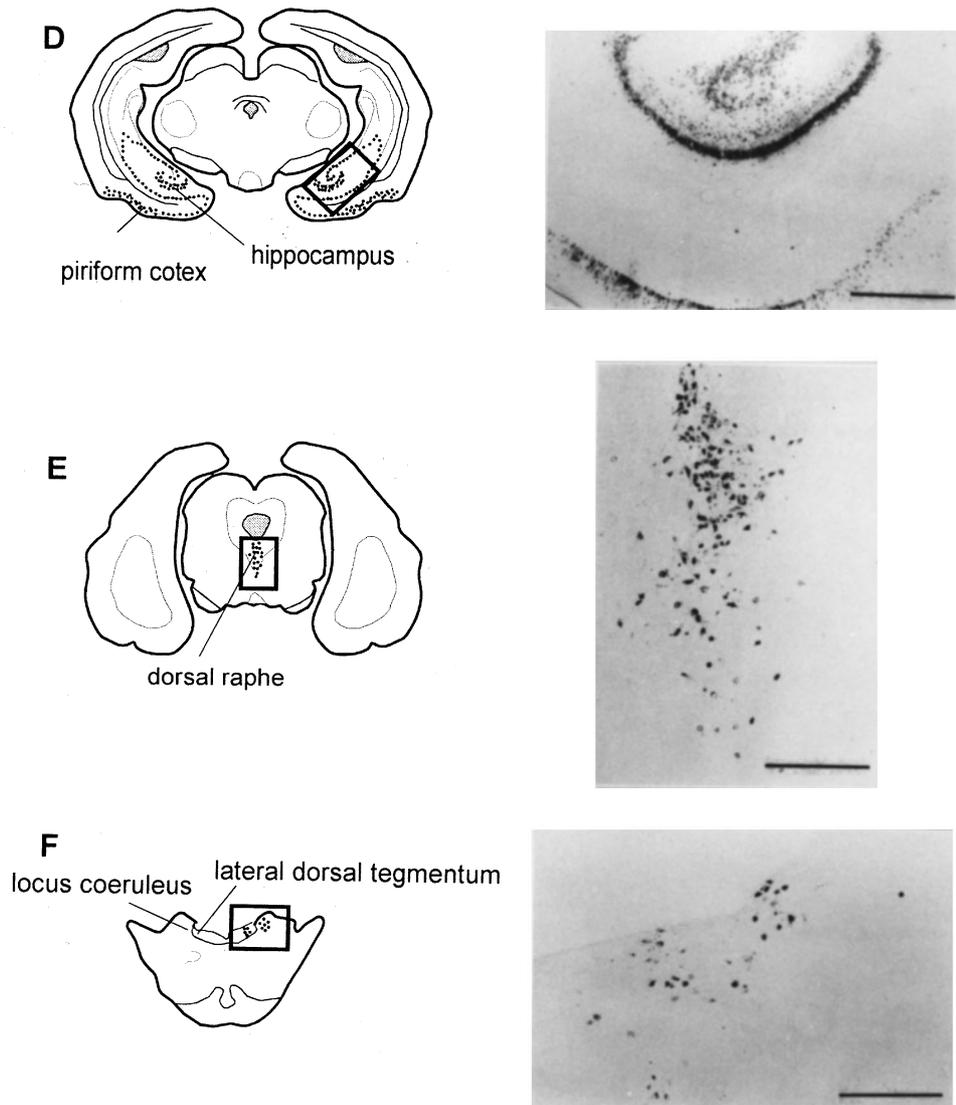
The location and extent of virus infection in neurons of the CNS and TG, as detected by immunocytochemistry, are summarized in Table 2. At 4 d.p.i., when the rabbits showed mild neurological signs (hyperaesthesia and head-twitching), viral antigens were detected in neurons of the glomerular, external plexiform, and mitral cell layers of the olfactory bulb (Figure 1A and Table 2). Additionally, the fibers of infected neurons in the external plexiform and mitral cell layers contained antigen (Figure 1A'). At 6 d.p.i., viral antigens were detected in all of the above areas



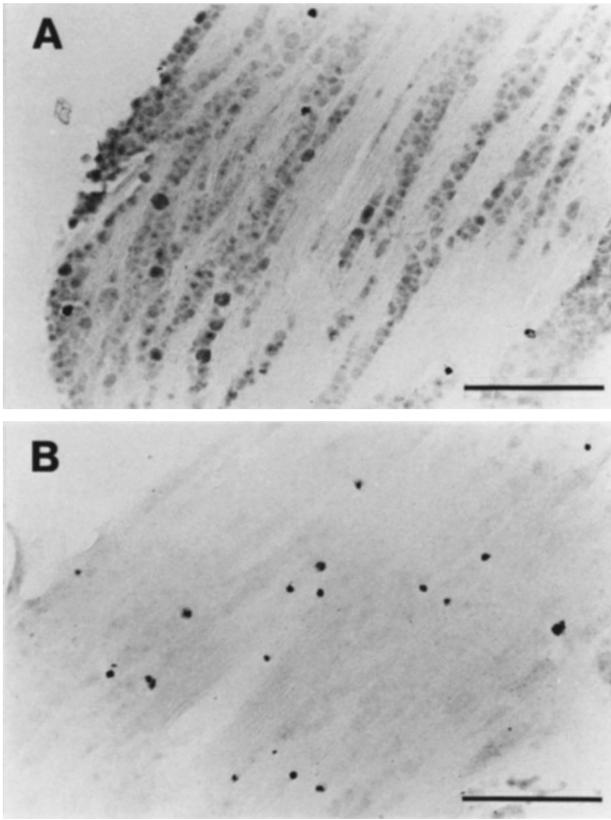
as well as in the inner granular layer (Figure 1B). More neurons over a greater area were affected in the main olfactory bulb.

The distribution of infected neurons after 8 d.p.i. is shown in Figure 2. At 8 and 9 d.p.i., virus-infected neurons were detected throughout the olfactory pathway. Many stained neurons (>150–500/field at 5× magnification) were present in the anterior olfactory nucleus and olfactory tubercle (Figure 2A), frontal/cingulate and piriform cortices (Figure 2A, B and C), amygdala (Figure 2C), and hippocampus (Figure 2D). In the frontal/cingulate and piriform/entorhinal cortices, neurons of the pyramidal and polymorph cell layers (layers 4 and 5 in frontal

and entorhinal cortices, and layers 2 and 3 in piriform and cingulate cortices) were infected. In the dentate gyrus and hippocampus, neurons in the granular and pyramidal cell layers, respectively, had the most staining. Neurons in the dorsal raphe (Figure 2E) also had dense staining; however, the number of neurons stained was comparatively lower (50–100 infected neurons/field) than in the above-mentioned structures. Positively stained regions in the locus coeruleus and the lateral dorsal tegmentum had approximately 5–10 infected neurons/field (Figure 2F and Table 2). In case of one rabbit (#10), 10–20 infected neurons/field (data not shown) were present in the central raphe



**Figure 2** Localization of BHV-5 specific antigen in the rabbit brain at 8 d.p.i. Schematic representation of the rabbit brain depicting the CNS distribution of BHV-5-positive neurons (black dots indicate infected areas). Frames indicate areas shown in photographs. Bright-field photomicrographs show immunostained neurons (indicated by dense black precipitate over the cell). (A) Lateral olfactory tubercle, frontal cortex and piriform cortex (B) cingulate cortex, (C) piriform cortex and amygdala, (D) hippocampus, (E) dorsal raphe, (F) locus coeruleus and lateral dorsal tegmentum. Scale bars: 1600  $\mu\text{m}$  (A and C), 400  $\mu\text{m}$  (B, D, E and F).



**Figure 3** Immunostained neurons in the trigeminal ganglion in the rabbit. (A) Infected neurons in trigeminal ganglion (TG) at 4 d.p.i. (B) Infected neurons in TG after 6 d.p.i. Note that the processes of infected neurons in the TG did not stain. Scale bar: 200  $\mu$ m.

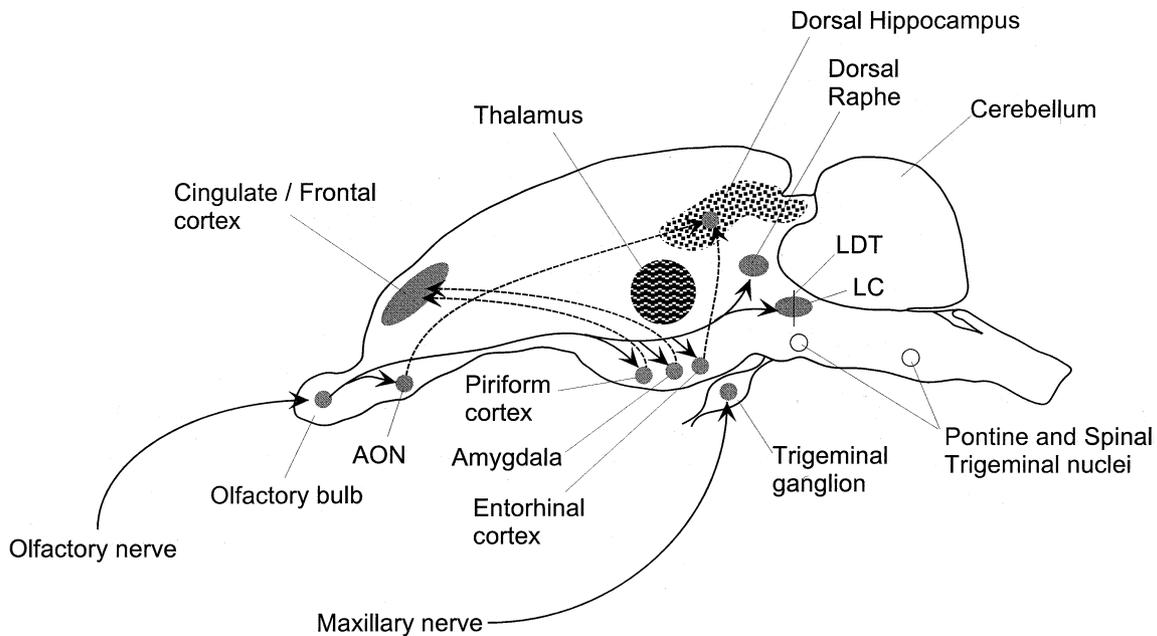
nucleus (medial part) and in the parabrachial nucleus. At 10 d.p.i., rabbit #16 had no stained neurons. However, at 12 d.p.i., rabbit #9 had a few stained neurons in the piriform/entorhinal cortices and amygdala. At 2, 4, 6, and 8 d.p.i., 3–10 infected neurons per field were detected within the trigeminal ganglion. The staining pattern in the trigeminal ganglion was intracellular; dendrites or axons of infected cells were not stained (see Figure 3). No stained neurons were present in the thalamus, hypothalamus, pontine and spinal nuclei of the trigeminal nerve, or cerebellum of any infected rabbits.

### Discussion

The results of this study indicate that invasion and sequential spread of BHV-5 within the rabbit brain, following intranasal inoculation, are predominantly via the olfactory pathway. Neurological signs occurred following viral invasion and the development of lesions in the third and fourth order neuronal structures of the olfactory pathway.

#### *Olfactory and trigeminal pathways*

The olfactory pathway includes multiple neuronal projections (Brodal, 1969; Jenkins, 1978; Lohman and Lammers, 1967). The first neuronal level consists of the olfactory receptor cells, which are located among the nonneuronal cells of the olfactory epithelium. The receptor cells of the olfactory epithelium are bipolar neurons whose



**Figure 4** Schematic of proposed route and viral spread in the olfactory and trigeminal pathways after intranasal instillation. Solid lines indicate the structure connected directly to the olfactory bulb. Broken lines indicate the structure connected indirectly to the olfactory pathway.

dendrites are in contact with the external environment (Frisch, 1967; Cuscheri and Bannister, 1975). These neurons are unique, because they are exposed directly to the environment and can incorporate protein instilled into the cavity by endocytosis (Kristensson and Olsson, 1971). They can be infected by neurovirulent viruses without prior replication in the surrounding epithelial cells (Lafay *et al*, 1991). Axons of these receptor neurons terminate in the glomerular layer of the main olfactory bulb (MOB) where they synapse with the dendrites of periglomerular cells, the tufted cells, and the mitral cells (second order neurons). The inner granular neurons (third neuronal level) make reciprocal synapses with the dendrites of mitral/brush cells (tufted neurons) (Halasz and Shepherd, 1983). In addition, the axons of both mitral and brush neurons leave the olfactory bulb and terminate in other structures of the brain, among which are the anterior olfactory nucleus (AON), lateral olfactory tubercle (LTu), piriform cortex, entorhinal cortex, amygdala, and dorsal raphe. These areas represent the third order neurons of the olfactory pathway. In addition, reciprocal projections from the AON, piriform cortex, amygdala, entorhinal cortex, dorsal raphe, lateral dorsal tegmentum (LDT), and locus coeruleus (LC) terminate in the olfactory bulb (Shipley *et al*, 1994).

The major inputs to the dentate gyrus and to the CA1 and CA3 regions of the hippocampus are from the entorhinal cortex (fourth order neuronal connections). The dentate gyrus and cells of CA3 do not project back to the entorhinal cortex. However, the dentate gyrus and CA3 region project back to the CA3 and CA1 regions, respectively. The CA1 region of the hippocampus projects back to the entorhinal cortices. Additionally, the CA region of the hippocampus projects to the AON, thus connecting the hippocampus and the dentate gyrus reciprocally and indirectly to the olfactory pathway (Amaral and Witter, 1994). Similarly, efferent projections from the cingulate/frontal cortex (fourth order neurons) project to the piriform cortex and amygdala, thus connecting them indirectly to olfactory structures (Zilles and Wree, 1994).

The trigeminal pathway also contains multiple neuronal levels (Jenkins, 1978). The main sensory innervation of the nasal mucosa is mediated by branches of the maxillary nerve, which represent the distal dendrites of pseudo-unipolar cells of the trigeminal ganglion (Getty, 1975). The unipolar neurons of the trigeminal ganglion represent the first neuronal level of the trigeminal pathway. Axons of these neurons extend to the principal (pontine) nucleus and spinal nucleus of the trigeminal nerve (second order neurons) located in the pons and medulla oblongata, respectively (Jenkins, 1978). Axons arising from the second order neurons extend into the thalamus and/or

cerebellum (third order neurons). Axons of the third order neurons in the thalamus extend into the cerebral cortex (Jenkins, 1978).

*BHV-5 invades the CNS via the olfactory pathway*  
The route of invasion of BHV-5 into the CNS is depicted in Figure 4. Following BHV-5 inoculation of the paranasal sinus of rabbits, immunocytochemically stained neurons containing virus specific antigens were present in the MOB (second order neurons) as early as 4 d.p.i. At 6 d.p.i., the number of stained neurons in the olfactory bulb increased, and viral antigen was present in deeper olfactory neurons. By 8 d.p.i. when the rabbits had severe neurological signs and histologic lesions, a substantial number of third and/or fourth order neurons in the AON, LTu, piriform/entorhinal cortex, amygdala, and dorsal raphe contained viral antigen. With the exception of LTu, all of these areas project to the MOB via efferent connections (Shipley *et al*, 1994). Thus, the virus appears to spread retrogradely to AON, piriform/entorhinal cortex, amygdala, and dorsal raphe but anterogradely to LTu (Figure 4). Immunostained neurons were also present in the LC and LDT (third and/or fourth order neurons). These two areas project to the MOB (Herrero *et al*, 1991; McLean *et al*, 1993). Thus, the virus spread retrogradely to these two areas (Figure 4). By 8–9 d.p.i., fourth order neurons in the frontal/cingulate cortices, dentate gyrus, and hippocampus contained viral antigen that most likely was spread from the AON, piriform/entorhinal cortex, and amygdala (Figure 4). The alternative route of CNS invasion, the trigeminal pathway, did not appear to be involved in this study. In the trigeminal pathway, 2–10 stained nerve cell bodies were present in the trigeminal ganglion from 2–8 d.p.i. The virus most likely reached these neurons by retrograde movement via the maxillary branch of the trigeminal nerve. However, further spread to the pontine and spinal trigeminal nuclei (second order neurons in the trigeminal pathway) of the pons and medulla, respectively, did not occur. In addition, the staining of TG neurons was restricted to the cell bodies only and not their processes. In contrast, staining in the olfactory pathway consistently occurred in both nerve fibers and nerve cell bodies. The failure to isolate virus from the TG and the lack of staining in nerve cell processes suggests that the virus did not replicate efficiently in the neurons and probably was not transported out of the trigeminal ganglion.

*Neurological signs correlate with the viral spread in the brain*

In this study, viral spread and replication correlated well with the progression of neurological signs. Mild neurological signs appeared at 4 and 6 d.p.i. and were associated with immunostaining of neurons in the main olfactory bulb (glomeruli,

external plexiform, mitral cells, and inner granular neurons). Virus could be isolated only from the olfactory bulbs, and no histopathological lesions were present. Severe neurological signs at 8 d.p.i. were associated with massive numbers of stained neurons in the piriform cortex, amygdala, cingulate cortex, and hippocampus. Virus was isolated in high titers, and histopathologic lesions consisting of neuronal necrosis and lymphocytic meningoencephalitis were prominent.

These data suggest that infection of the anterior olfactory nucleus, piriform and cingulate/frontal cortices, amygdala, hippocampus, and dentate gyrus is responsible for the development of severe neurological signs. The hippocampus, amygdala, and piriform/entorhinal cortices are involved when limbic seizures are induced by the injection of a cholinergic muscarinic antagonist (Motte *et al*, 1998). The hippocampus also could be the primary structure involved in the generation of measles virus-induced seizures (Urbanska *et al*, 1997). The disruption of cellular metabolism by viral replication in these regions may have contributed to the seizures observed in these rabbits (Halliday, 1975).

When an animal shows seizures, that animal usually would die within 8–12 h. If an animal was found to have severe seizures, it was euthanized within 2–3 h (as mandated by our animal care committee). Thus, the animals killed at 10 and 12 days did not show seizures at early times, but showed mild neurological signs exemplified by hyperaesthesia, trembling, head twitching, etc. In our previous paper, we reported that 20–30% of the animals show only a mild form of infection and usually survive (Chowdhury *et al*, 1997). In histopathology, the brains of these animals would show immune response to virus infections characterized by perivascular cuffing, nodular gliosis, etc. the virus isolation and immunohistochemistry results may or may not be positive depending on virus load. It is possible that in these animals the virus has gone latent in the CNS—this question was not investigated here. Thus, the question of whether virus has gone latent in the animals that survived > 10 days has not been addressed in this paper.

*Factors affecting neuroinvasiveness in olfactory and trigeminal pathways for other neurovirulent viruses*

Other neurovirulent viruses invade the CNS following intranasal deposition via the olfactory pathway, e.g., vesicular stomatitis virus (VSV) (Lundh *et al*, 1987, 1988) and murine hepatitis virus (MHV) (Barthold, 1988) or via both the olfactory and trigeminal pathways, e.g., herpes simplex virus type 1 (HSV1) (McLean *et al*, 1989; Barnett *et al*, 1994) and pseudorabies virus (Barbic *et al*, 1994; Kritas *et al*, 1994a,b). Several factors may determine whether the CNS will be invaded by a neurovirulent virus along cranial or peripheral nerves. These include a primary cycle of virus

replication in the innervated area, viral attachment to axonal or dendritic terminals of the primary neurons, multiplication and vectorial transport of viral components in the nerve cells and their processes, and restriction of virus spread by the host animal defense mechanisms against the infection. Affinity for the olfactory or respiratory epithelium and the nature of viral budding are critical factors in the differential neuroinvasive properties of vesicular stomatitis virus (VSV) and Sendai virus (SV) (Lundh *et al*, 1987). Neuroinvasive VSV causes extensive infection of the olfactory epithelium with minimal involvement of the respiratory epithelium. Conversely, nonneuroinvasive SV causes extensive infection of the respiratory epithelium with only minimal infection of the olfactory mucosa. Differences in viral budding account for some neuroinvasive potential. The VSV buds from the basolateral surfaces of supporting cells and olfactory neurons, but not from their apical surfaces or the ciliated bulbous endings of the olfactory neuron dendrites, thus favoring neuroinvasion. The SV buds only from the apical surface of respiratory epithelial cells and is released into the air passage without invasion into the olfactory bulbs (Lundh *et al*, 1987).

Following intranasal inoculation, swabs obtained from the nasal turbinates and nasal vestibular regions yielded higher quantities of BHV-1, than of BHV-5 (Chowdhury, unpublished data). However, only BHV-5 invades the CNS via the olfactory pathway. When inoculated intracerebrally, both BHV-1 and 5 are equally neurovirulent (Chowdhury, unpublished results). This implies that differences exist in BHV-1 and BHV-5 interactions with the olfactory receptor neurons that may be important in the neuropathogenesis of BHV-5. Alternatively, the viruses may differ in their ability to spread anterogradely from the olfactory receptor neurons (first order neurons) to the olfactory bulb (second order neurons). However, the failure of BHV-5 to spread anterogradely from TG to pontine and spinal nuclei is not consistent with this assumption.

In comparison to other sensory pathways, the olfactory nervous pathway exhibits several unique features. First, the olfactory receptor cells (first order neurons) are the only known neurons that are replaced in postnatal life (reviewed in Cormack, 1987). Additionally, the olfactory receptor cells provide a direct connection between the brain and the external environment, a feature lacking in other neural pathways, including the trigeminal pathway where external tissues or synapses interrupt the route towards the CNS.

*Natural pathogenesis of BHV types 1 and 5*

BHV-1 and BHV-5 are related antigenically and genetically, however, they differ markedly in their ability to cause neurological disease in calves

(Belknap *et al*, 1994). BHV-1 causes respiratory and genital infections and occasionally abortion and enteritis in cattle (Wyler *et al*, 1989). BHV-1 has been divided into two subtypes based upon DNA restriction profiles and pathogenic properties into the respiratory, abortigenic subtype (IBR, BHV-1.1) and the non-abortigenic, genital subtype (IPV, BHV-1.2). BHV-5, also known as bovine encephalitic herpesvirus (BEHV), is the causative agent of a fatal meningo-encephalitis in calves (Engels *et al*, 1987; Bulach and Studdert, 1990). Calves experimentally inoculated, intranasally, with BHV-5 showed evidence of extensive widespread meningo-encephalitis and neural necrosis and viral replication in the brain. In contrast, BHV-1 inoculated calves had no brain lesions (Belknap *et al*, 1994). In previous work, we have demonstrated that in the rabbit model, BHV-5 was capable of producing a fatal meningo-encephalitis with severe neurological signs and BHV-1 was not neurovirulent in rabbits under similar experimental conditions (Chowdhury *et al*, 1997). Based upon the results of the present paper and those obtained previously, we suggest that BHV-1 and BHV-5 differ in their affinity for olfactory receptor neurons (ORN). BHV-5 may have an affinity for the specific receptors on the first order olfactory neurons, whereas BHV-1 does not. In addition, our results suggest that the TG is not important for the neuropathogenic effects of BHV-5 in the rabbit model. If these assumptions are true, then the mechanism by which BHV-5 invades the CNS may be unique and different from that of VSV, HSV-1, and PRV because these viruses penetrate the CNS via TG pathway, in addition to the olfactory pathway.

## Materials and methods

### *Virus and cell cultures*

The BHV-5 strain (TX-89) isolated from a case of viral encephalitis (d'Offay *et al*, 1993) was used for the study. Stocks of virus were prepared in Madin-Darby bovine kidney (MDBK) cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Virus was titrated by standard plaque assay, aliquoted, and frozen at  $-80^{\circ}\text{C}$ .

### *Animals*

Sixteen, 4-week-old New Zealand White rabbits (500–600 g body weight) were used (Myrtles Rabbitry, Thomson Station, TN, USA). Rabbits were maintained in laboratory isolation cages in our vivarium throughout the experiment with food and water freely available. All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

### *Rabbit inoculation*

Rabbits were anesthetized by intramuscular injection of ketamine (10 mg/kg) and xylazine (5 mg/kg)

and infected intranasally with BHV-5 virus ( $2 \times 10^7$  PFU/0.3 ml per nostril) (Chowdhury *et al*, 1977). Following infection, rabbits were observed three times daily for clinical signs (Chowdhury *et al*, 1997). At least, two rabbits were sacrificed at 2, 4, 6, 8, 10 and 12 d.p.i. or when they developed severe neurological symptoms. At each time period, one rabbit was used for virus isolation (left brain hemisphere) and histopathological examination (right brain hemisphere) (rabbits #2, 4, 6, 7, 8, 11, 13, 14), and the other rabbit was perfused (rabbits #1, 3, 5, 9, 10, 12, 15, 16) for immunocytochemistry.

### *Virus isolation*

Nasal swabs were obtained at each time point for virus isolation. A plain cotton-tip swab was inserted into each nasal vestibule and nasal turbinate and rotated three times against the mucosa. Virus was eluted from the swabs in 1 ml of sample medium for 1 h at  $4^{\circ}\text{C}$ , filtered, and assayed by standard plaque assay.

The left brain hemisphere was dissected into six regions: the olfactory bulb; midbrain and diencephalon (thalamus and hypothalamus); anterior cortex (frontal area, olfactory tract, cingulate cortex, and the anterior portion of the piriform cortex); posterior cortex (temporal area, piriform area, entorhinal area, parietal area, occipital area, and hippocampus); pons and medulla; and cerebellum. The left trigeminal ganglion and each of the six regions were homogenized separately for virus isolation (Chowdhury *et al*, 1997). All cultures without evidence of cytopathic effect were re-passaged to confirm the absence of the virus.

### *Immunocytochemistry*

Virus-specific antigens were detected using immunocytochemistry (Weiss and Chowdhury, 1998). Briefly, each rabbit was deeply anesthetized and transcardially perfused, using a peristaltic pump, with 500 ml of heparinized (3 IU/ml) isotonic saline, followed by 2 l of 10% buffered neutral formalin. The whole brain and trigeminal ganglion were removed, fixed overnight in 10% buffered neutral formalin, and transferred to 20% sucrose in PBS for cryoprotection. The trigeminal ganglia were embedded in 10% gelatin. The tissues were frozen on dry ice, and serial  $40 \mu\text{m}$  sections were made using a sliding microtome (American Optical Corp. model 860, Buffalo, NY, USA). Every sixth serial section from the brain and every section from the trigeminal ganglion were processed for immunocytochemistry. Endogenous peroxidase was inactivated by incubation with 0.3% hydrogen peroxide for 30 min followed by five washes (5 min/wash) with PBS containing 2% Triton X-100 (PBS-TX). After 30 min incubation in 5% inactivated horse serum in PBS-TX, the tissues were incubated overnight at  $4^{\circ}\text{C}$  with polyclonal bovine serum directed against BHV-5 (1:10 000 in PBS-TX)

(Chowdhury *et al*, 1997). Sections then were washed five times in PBS-TX and incubated for 3 h at room temperature with anti-bovine biotinylated antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:400 in PBS-TX. After being washed three times with PBS-TX, tissues were processed with the avidin-biotin-peroxidase procedure using Vectastain reagents (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase was visualized by incubation with 0.05% (wt/vol) diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) and 0.01% (vol/vol) hydrogen peroxide in 175 mM sodium acetate, 10 mM imidazole, pH 7.2 (A/I buffer) for 2 min. The reaction was stopped by washing with A/I buffer. Immunocytochemically stained sections were mounted on gelatin-coated slides, air dried, rinsed in distilled water, and dried overnight. The tissue was dehydrated in ethanol and cleared in xylene, and a coverslip was added with DPX mountant (Fluka, Ronkonkoma, NY, USA). Positive staining was detected by the presence of dense black precipitate over the cell.

To confirm immunocytochemical specificity, the primary antibody was eliminated from the protocol. Removal of the primary antibody completely eliminated positive immunocytochemical staining.

#### Histopathology

Tissues were fixed in 10% neutral buffered formalin for a minimum of 24 h. Tissue sections from the regions of interest then were dehydrated in sequential alcohol washes and embedded in paraffin. Four, 6 µm sections were cut, mounted on slides, stained with hematoxylin and eosin, overlaid with a coverslip, and observed microscopically for morphologic alterations.

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