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Effect of Olfactory Bulb Ablation on Spread of a Neurotropic Coronavirus into the Mouse Brain

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Summary

Previous results suggested that, after intranasal inoculation, mouse hepatitis virus (MHV), a neurotropic coronavirus, entered the central nervous system (CNS) via the olfactory and trigeminal nerves. To prove this hypothesis, the effect of interruption of the olfactory pathway on spread of the virus was studied using in situ hybridization. Unilateral surgical ablation of this pathway prevented spread of the virus via the olfactory tract on the side of the lesion. MHV RNA could be detected, however, at distal sites on the operated side, indicating that the virus spread via well-described circuits involving the anterior commissure from the control (intact) side of the brain. Viral transport via the trigeminal nerve was not affected by removal of the olfactory bulb, showing that the surgical procedure was specific for the olfactory pathway. These results prove conclusively that MHV gains entry to the CNS via a transneuronal route, and spreads to additional sites in the brain via known neuroanatomic pathways.

Mouse hepatitis virus (MHV),[1] a member of the coronavirus family, causes several diseases in small rodents, including hepatitis, gastroenteritis, and encephalomyelitis (1). Strain JHM (MHV-JHM) is a prototypic neurotropic strain and causes acute encephalitis as well as acute and chronic demyelinating disease in rats and mice (2–7).

Suckling mice inoculated with MHV-JHM invariably develop a fatal encephalitis. This disease can be prevented if mice are protected with an infusion of mAb or if they are nursed by immunized dams. Similarly, mice inoculated with an attenuated strain of virus do not develop the acute encephalitis (8–14).

Suckling C57BL/6 mice inoculated intranasally with MHV-JHM and nursed by immunized dams do not develop acute encephalitis, but a substantial fraction (40–90%) develop hindlimb paralysis with demyelination present on histological examination (12). Previous studies have delineated the spread of virus after intranasal inoculation. Virus enters the brains of both susceptible and maternal antibody-protected mice via the olfactory and trigeminal nerves. In mice that are susceptible to the acute encephalitis, virus first travels along known neuroanatomic pathways and then spreads to involve other portions of the brain as the mouse develops a panencephalitis (15).

In mice protected by maternal antibody, virus also enters via the olfactory and trigeminal nerves. Virus does not, however, invade other parts of the brain, but remains confined to the neuroanatomic connections of the trigeminal and olfactory nerves (15). Virus can be detected at 15 d post-infection in the anterior spinal cord of asymptomatic mice, at the same location as in mice that later develop hindlimb paralysis (16). Virus most likely spreads to this part of the spinal cord from the trigeminal nuclei via the reticular formation and the reticulospinal tract (16).

These experiments all suggest strongly that MHV-JHM enters the central nervous system (CNS) via the olfactory and trigeminal nerves after intranasal inoculation. To prove conclusively that virus spreads transneuronally into the CNS, viral spread was analyzed after unilateral surgical ablation of the olfactory bulb. Surgical bulbectomy should prevent viral entry via the olfactory nerve, but should not prevent spread via the bloodstream or via cerebrospinal fluid. In addition, spread along the ipsilateral trigeminal nerve, the other site of MHV entry into the CNS, should not be affected by removal of the olfactory bulb.

Materials and Methods

Animals and Virus. MHV-free C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used in all studies. MHV-JHM, originally obtained from Dr. S. Weiss (University of Pennsylvania, Philadelphia, PA), was plaque purified and grown as previously described (12).

Surgery. 28 4-wk-old mice were anesthetized with ketamine (200 mg/kg). The hair over the frontal and nasal bones were removed with Nair (Carter Products, Inc., New York, NY), and lidocaine (1%) was administered subcutaneously. Unilateral bulbectomy was performed essentially as described by Whitten (17). A 1-cm

[1] *Abbreviations used in this paper:* CNS, central nervous system; MHV, mouse hepatitis virus.

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incision was made in the skin ~0.5 cm caudal to the eyes. A small hole was made in the frontal bone overlying the olfactory bulb and dura, and the left olfactory bulb was gently aspirated. The skin was closed with a single suture. The surgical survival rate was 71% (20/28). An additional three mice died over the next 2 d, leaving 17 mice that could be evaluated. After the mice were killed, the completeness of the bulb removal was estimated to be 90% or better by visual inspection, with only occasional, minimal destruction of other parts of the brain.

After 24 h, 9×10^4 PFU MHV-JHM were inoculated into each nostril after light anesthesia with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, NJ). Brains were removed from mice at 4 d (six mice) or 5 d (11 mice) post-infection, and individual halves were prepared for in situ hybridization as previously described (18).

In Situ Hybridization. In situ hybridization was performed on frozen sections using a ^{35}S-labeled antisense RNA probe, as previously described (18), except that slides were pretreated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO) before section placement. The RNA probe was complementary to MHV genes 5 and 6 and to 200 bases of genes 4 and 7. Slides were analyzed both by exposure to x-ray film (Eastman Kodak, Rochester, NY) and by emulsion autoradiography (Kodak NTB-2 track emulsion). A negative (uninfected brain) and a positive (brain from a mouse with acute encephalitis) control were analyzed in each experiment. No signal was detected by film autoradiography when sections from uninfected mice were analyzed.

Results

Intranasal Inoculation of 4-wk-old Mice with MHV-JHM. After 10-d-old C57BL/6 mice were inoculated intranasally with MHV-JHM, viral RNA was detected in the olfactory and trigeminal nerves and their intermediate connections (15). To prove that MHV-JHM spreads transneuronally, we attempted unilateral ablation of the olfactory bulb in these suckling mice. In preliminary experiments, we found that denervation by surgical methods was very difficult due to the small size of these animals. On the other hand, 4-wk-old mice, which are significantly larger, survived surgery and anesthesia, and the absence of fully calcified skulls facilitated the surgery. According to published reports, MHV causes an acute nasoencephalopathy in weanling and adult mice after intranasal inoculation (19–21), so it was anticipated that MHV-JHM would readily enter the CNS of 4-wk-old mice after this route of inoculation.

To verify that this was true, the brains of 4-wk-old C57BL/6 mice were analyzed by in situ hybridization at several times after intranasal inoculation (Fig. 1). At 5 d post-infection, viral RNA could readily be detected in parts of the brain connected anatomically to the olfactory and trigeminal nerves, such as the olfactory bulb, the fornix, the hypothalamus, the mesencephalic nucleus of the trigeminal nerve, and the reticular nuclei (Fig. 1 *B*). By 8 d post-infection, mice showed signs of severe encephalitis, including irritability, hunching, and ruffled fur. Viral RNA was detected diffusely throughout the brain, with very prominent labeling in the reticular formation (Fig. 1 *C*).

Effect of Surgical Ablation of Olfactory Bulb The above results suggested that MHV-JHM entered the CNS of 4-wk-old mice, as in suckling mice, via the olfactory and trigeminal nerves, and that spread to other parts of the brain occurred via neuroanatomic connections of these two nerves. If this interpretation is correct, surgical interruption of one of the neuroanatomic pathways should prevent viral spread. The olfactory pathway is amenable to surgical intervention at the level of the nasal epithelium, the olfactory nerve, or the olfactory bulb. Removal of the olfactory bulb is readily accomplished in 4-wk-old mice, and this operation was performed in order to interrupt spread of MHV-JHM via the olfactory pathway. In addition, only one olfactory bulb from each mouse was removed, so that the intact bulb served as an internal control for virus spread. This surgical procedure should have no effect on the spread of the virus via the trigeminal nerve on either side of the brain.

Unilateral olfactory bulbectomy was performed on 28 4-wk-old C57BL/6 mice, and, as described in Materials and Methods, 17 mice could be evaluated. Consistent results were obtained with all mice. At 4 d postinfection, viral RNA could be detected by in situ hybridization in the olfactory bulb,

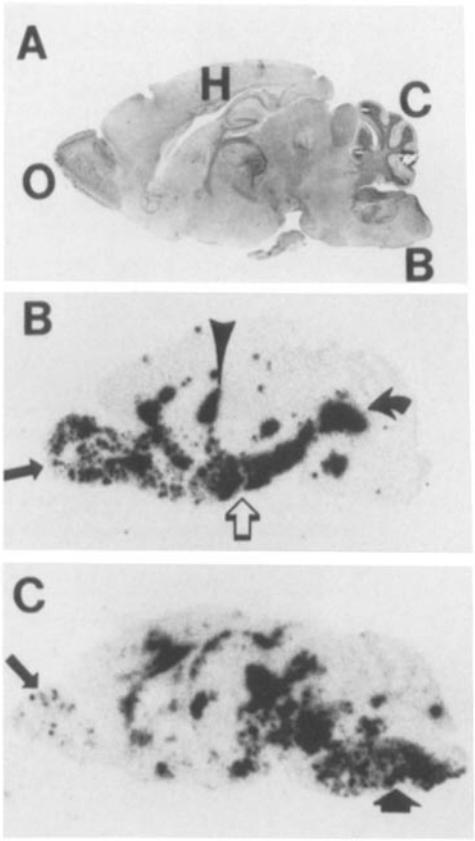

Figure 1. Localization of MHV-JHM in 4-wk-old C57BL/6 mice. Mice were inoculated intranasally with MHV-JHM. Brains were removed and analyzed by in situ hybridization and film autoradiography as described previously (18). (*A*) Frozen sagittal section stained with hematoxylin and eosin to show orientation of brain. (*O*) olfactory bulb; (*H*) hippocampus; (*C*) cerebellum; (*B*) brainstem. (*B*) 5 d post-infection. (*C*) 8 d post-infection. (*Closed arrow*) Olfactory bulb; (*arrowhead*) fornix; (*open arrow*) hypothalamus; (*curved arrow*) mesencephalic nucleus of the trigeminal nerve; (*short, wide arrow*) reticular formation.

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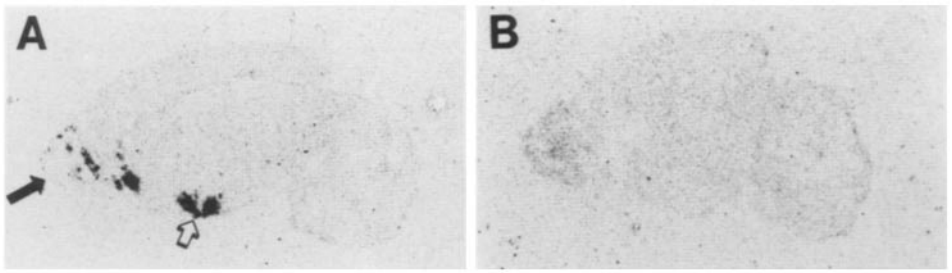

Figure 2. Location of virus 4 d post-infection in mice after unilateral olfactory bulb ablation. Sagittal sections were prepared from a mouse inoculated intranasally and analyzed by in situ hybridization. (*A*) Control (intact) side of brain; (*B*) side of brain from which bulb was removed. (*Closed arrow*) Olfactory bulb; (*open arrow*) nucleus of the diagonal band of Broca.

anterior olfactory nuclei, and lateral olfactory tract on the control side of the brain, with no virus present on the side of the brain from which the bulb had been removed (Fig. 2). No labeling was evident in the mesencephalic nucleus of the trigeminal nerve when analyzed by film autoradiography, but a few small foci of viral RNA could be detected in some mice on both sides by emulsion autoradiography and light microscopy (data not shown). These results are consistent with previous data showing that MHV-JHM was transported more rapidly via the olfactory than trigeminal nerves (15).

By 5 d post-infection, viral RNA could be detected in several locations on both the control and experimental sides of the brain. In particular, the mesencephalic nucleus of the trigeminal nerve was labeled on both sides (Figs. 3, 4), indicating that the surgical procedure did not affect transport of virus via the trigeminal nerve. In marked contrast, no viral RNA could be detected in the most anterior part of the brain from which the olfactory bulb had been removed, but was clearly present in this region in the control hemisphere.

In addition, viral RNA could be detected bilaterally in more distant connections of the olfactory system, such as the nucleus of the diagonal band of Broca, the fornix, the mammillary nuclei, and the limbic cortex. This spread of virus on the operated side most likely occurred via olfactory fibers known to cross to the opposite side of the brain via the anterior commissure. To show that viral RNA crossed from the olfactory bulb and nuclei on the control side to the operated side via the anterior commissure, the latter structure was analyzed for the presence of viral RNA. Viral RNA was present in the anterior commissure on both the control (Fig.

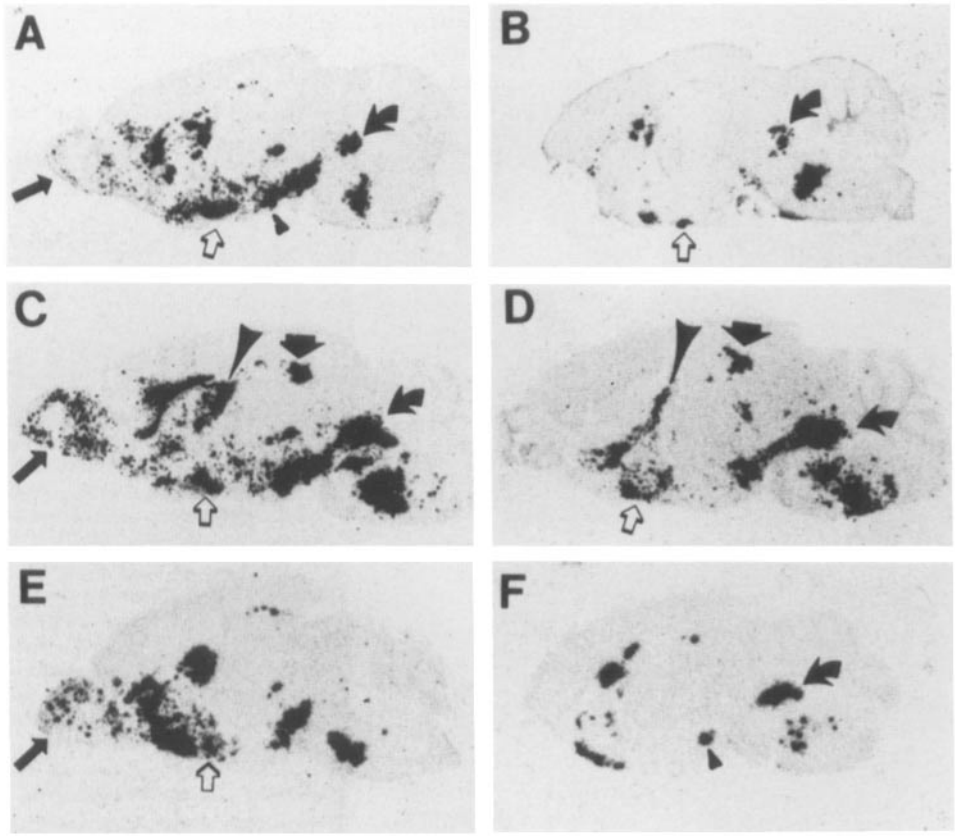

Figure 3. Location of virus 5 d post-infection in mice after unilateral olfactory bulb aspiration. Sagittal sections were prepared from mice inoculated intranasally and analyzed by in situ hybridization. Brains from three different mice are shown in the figure (*A* and *B*; *C* and *D*; *E* and *F*). (*A*, *C*, and *E*) Control (intact) side of brain; (*B*, *D*, and *F*) side of brain from which olfactory bulb was removed. (*Straight arrow*) Olfactory bulb; (*open arrow*) nucleus of the diagonal band of Broca; (*large arrowhead*) fornix; (*small arrowhead*) mammillary nuclei; (*short, wide arrow*) limbic cortex; (*curved arrow*) mesencephalic nucleus of the trigeminal nerve.

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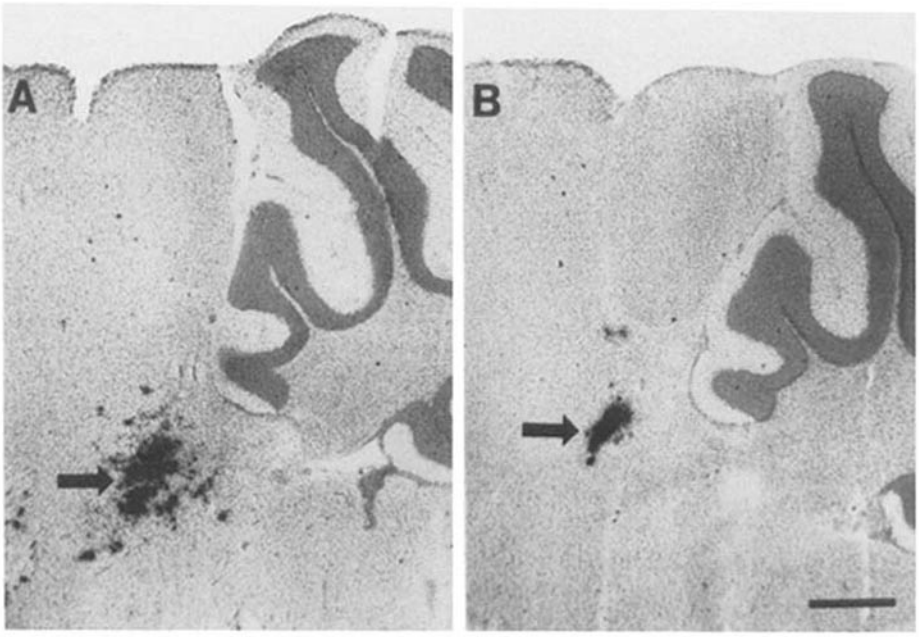

Figure 4. Microscopic localization of MHV-JHM in the mesencephalic nucleus of the trigeminal nerve. Sagittal sections were prepared 5 d post-infection from mice inoculated intranasally and analyzed by in situ hybridization and emulsion autoradiography. (*A*) Brainstem from control (intact) side; (*B*) brainstem from side from which olfactory bulb had been removed. The mesencephalic nuclei (*arrow*) on both sides of the brain are labeled to nearly the same extent, in contrast to the results shown in Fig. 5. Magnification bar, 0.5 mm.

5, *A* and *C*) and experimental (Fig. 5, *B* and *D*) sides of the brain.

Discussion

After intranasal inoculation, the neurotropic coronavirus MHV-JHM is readily detected in parts of the brain that are connected to the olfactory and trigeminal nerves, both of which innervate the nose. Previous virological, immunohistochemical, and in situ hybridization studies all suggested that virus spread transneuronally and not via blood or via cerebrospinal fluid (15, 16, 19–21). In addition, the presence of maternal antibody did not affect initial entry of MHV into the brain, consistent with spread via nerves (15). Transection of the nerve in question will prevent transneuronal movement, but not affect other routes of spread and thus provide definitive information as to the method of spread.

Unilateral destruction of the olfactory bulb clearly prevented ipsilateral spread of MHV-JHM via the olfactory pathway, but did not affect spread via the control (intact) olfactory

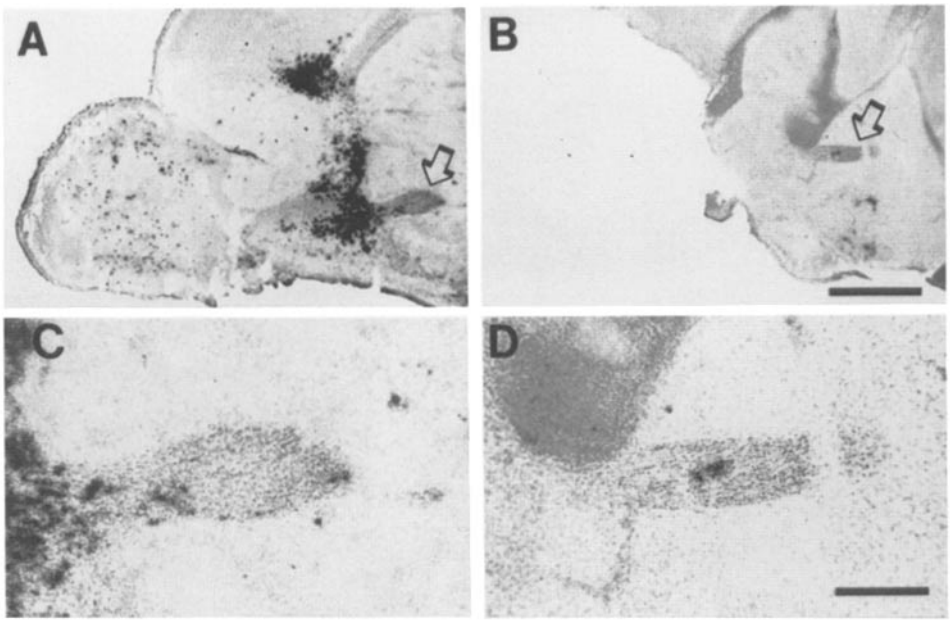

Figure 5. Microscopic localization of MHV-JHM in anterior commissure. Sagittal sections were prepared from mice inoculated intranasally and analyzed by in situ hybridization and emulsion autoradiography. (*A* and *B*) Anterior areas of the brain are shown, with greater labeling apparent on the control (intact) side (*A*) as opposed to the operated side (*B*). The anterior commissure (*arrow*) is shown at higher magnification in control side (*C*) and operated side (*D*). Note the lack of label anterior to the commissure in *B* and *D* as compared with *A* and *C*. Magnification bar, (*A* and *B*) 1 mm; (*C* and *D*) 0.25 mm.

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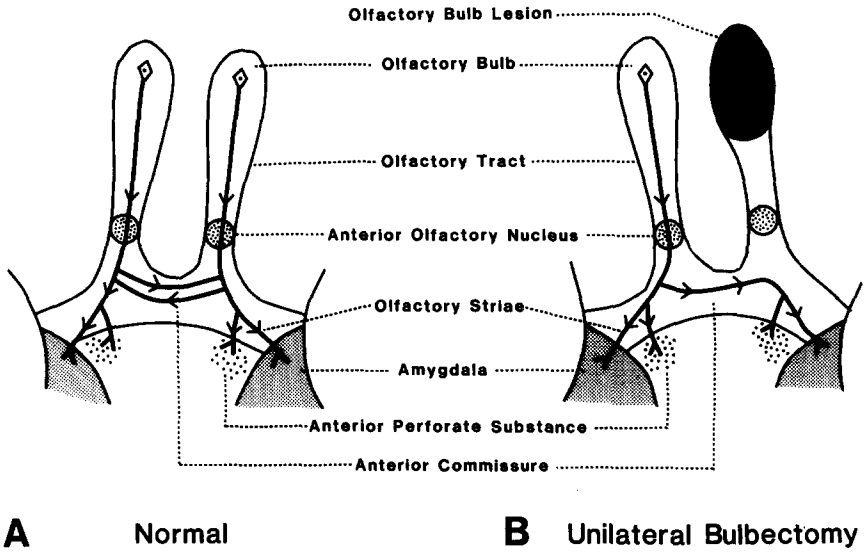

Figure 6. Schematic drawing of route of spread of virus through the mouse olfactory system. (*A*) MHV-JHM enters the CNS via the olfactory bulb and spreads via the olfactory tract to the olfactory striae. Some virus crosses to the opposite side of the brain via the anterior commissure, to enter the contralateral olfactory striae. Virus spreads via the medial and lateral olfactory striae to the prepyriform cortex and amygdala. (*B*) After unilateral destruction of the olfactory bulb, virus crosses from the control (intact) olfactory tract to the olfactory striae on the operated side via the anterior commissure.

bulb or via the trigeminal nerve. MHV-JHM RNA could be detected, however, on the operated side at sites more distally connected to the olfactory system, such as the nucleus of the diagonal band of Broca, the fornix, the mammillary nuclei, and the limbic cortex. This spread most likely occurs via fibers from the olfactory tract on the control side, which are known to cross to the opposite side of the brain via the anterior commissure (Fig. 6). From there, they project upon the anterior perforated substance, the prepyriform cortex, and the amygdala. From these sites, connections are established with other parts of the olfactory/limbic system.

Little is known about the exact mechanism by which MHV is transported along nerves. Herpes simplex virus appears to undergo fast axonal transport in both anterograde and retrograde directions, dependent on whether the virus is inoculated intradermally or intramuscularly (22, 23). Similarly, in studies with the type 3 strain of reovirus, Tyler et al. (24) showed that, after hindlimb injection, virus spread transneuronally to the spinal cord via microtubule-associated fast axonal transport. MHV-JHM cannot be detected in the mesencephalic nucleus of the trigeminal nerve until 3–4 d postinfection, but since virus is believed to replicate in the nasal epithelium before spread (19, 21), it is not possible to draw any conclusions about the rate of viral movement within the trigeminal nerve. By analogy with other viruses, however, MHV most likely spreads transneuronally via fast axonal transport.

Although the basis for the neurotropism of MHV-JHM is not well understood, the spike glycoprotein (S) has been shown to be a major determinant in the ability of different strains of MHV to infect neurons and glia (10, 11, 13, 25, 26). This tropism is not absolute, however, since the A59 strain of MHV is unable to replicate efficiently in neurons in vitro (27), but is still able to enter the mouse CNS via the olfactory and trigeminal nerves (15). A cell receptor for MHV has been partially characterized (28); determination of the viral receptor on neurons and glia will be important in elucidating the basis for the neurotropism of this virus.

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