JOURNAL OF VIROLOGY, July 1985, p. 158–162
0022-538X/85/070158-05$02.00/0

Vol. 55, No. 1

Pathways of the Early Propagation of Virulent and Avirulent Rabies Strains from the Eye to the Brain

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Received 18 October 1984/Accepted 11 March 1985

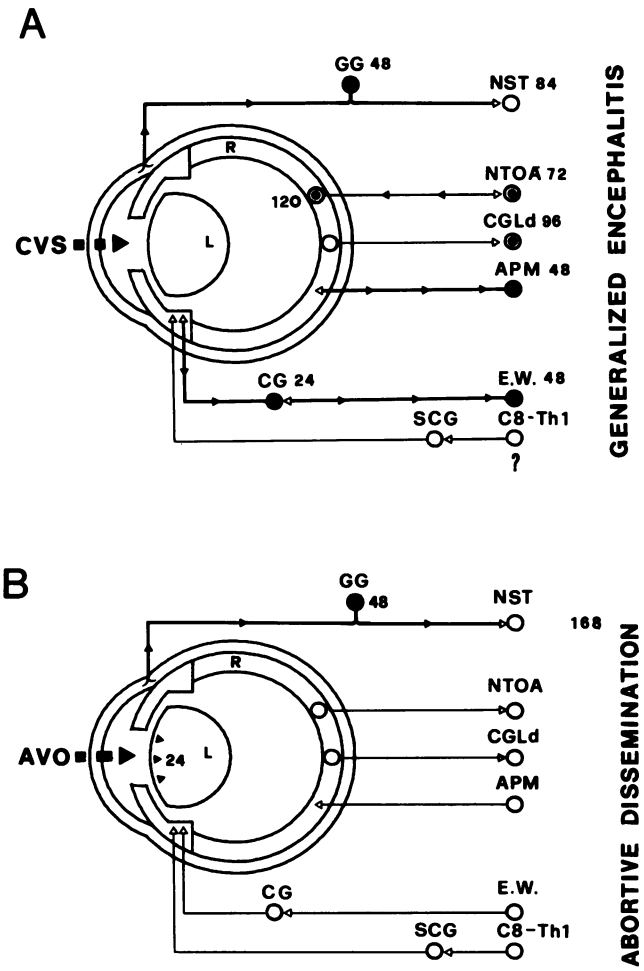
Penetration of the central nervous system of the adult rat by the CVS strain of rabies virus and its two avirulent derivatives Av01 and Av02 has been studied by inoculation of the virus into the anterior chamber of the eye. The primary sites of penetration of CVS were (i) the intraocular parasympathetic oculomotor fibers, (ii) the retinopetal fibers of pretectal origin, and (iii) the intraocular fibers of the ophthalmic nerve. The mutant strains, however, lost the capacity to invade the two former groups of fibers, although their penetration into the trigeminal system was not impaired. Neither strain CVS nor the mutants infected primarily the intraocular adrenergic terminals and the optic nerve. Mutant strains, but not CVS, were able to infect the lens. These results indicate that the cholinergic receptor may not be the only receptor for rabies virus and that rabies virus is conveyed in the nervous system by retrograde axoplasmic flow. Strain CVS spread throughout the brain and propagated eventually back to the retina. The mutants penetrated the brain as well, but the infection was slow, involved different cerebral structures, and cleared up completely in 3 weeks, probably because of an efficient immune response.

In an attempt to understand the molecular basis of rabies virus virulence, we have studied avirulent mutants of the CVS strain isolated from the survivors of neutralization with appropriate monoclonal antibodies (4). These mutants failed to kill adult animals irrespective of the dose and the route of inoculation (2, 3). The mutation has involved the viral glycoprotein where arginine 333 was replaced by glutamine or glycine (13, 14). By using the same procedure, other mutants of the CVS and ERA strains have been isolated presenting the same amino acid substitution. No other mutation in the glycoprotein has been detected (5).

The immune response of animals inoculated with the avirulent mutant Av01 of the Orsay collection or with the CVS parental strains was investigated. It has been shown that Av01 strongly potentiates the early phase of the interferon production and leads also to a high activity of natural killer and cytotoxic T cells. The levels of neutralizing antibodies in response to both the virulent and avirulent strains were developing similarly up to 1 week when the animals inoculated with the CVS died (1, 8). The high stimulation by Av01 of some parameters of the immune response was probably not the only effect of the mutation which led to a decreased pathogenicity. Preliminary observations in nude mice have suggested that the penetration and intracerebral propagation of virus could be affected as well. This hypothesis was tested by using a delimited and relatively well-known route of penetration of the central nervous system, namely, inoculation into the anterior chamber of the eye (6).

MATERIALS AND METHODS

A 10-μl portion of viral suspension (4×10^5 PFU) of CVS, Av01, or Av02 was injected under deep ether anesthesia into one eye of adult Wistar rats, using a syringe with a very thin needle. In general, the animals inoculated with CVS died within 1 week, whereas those inoculated with the mutants showed only transient behavioral anomalies and a 10 to 15%



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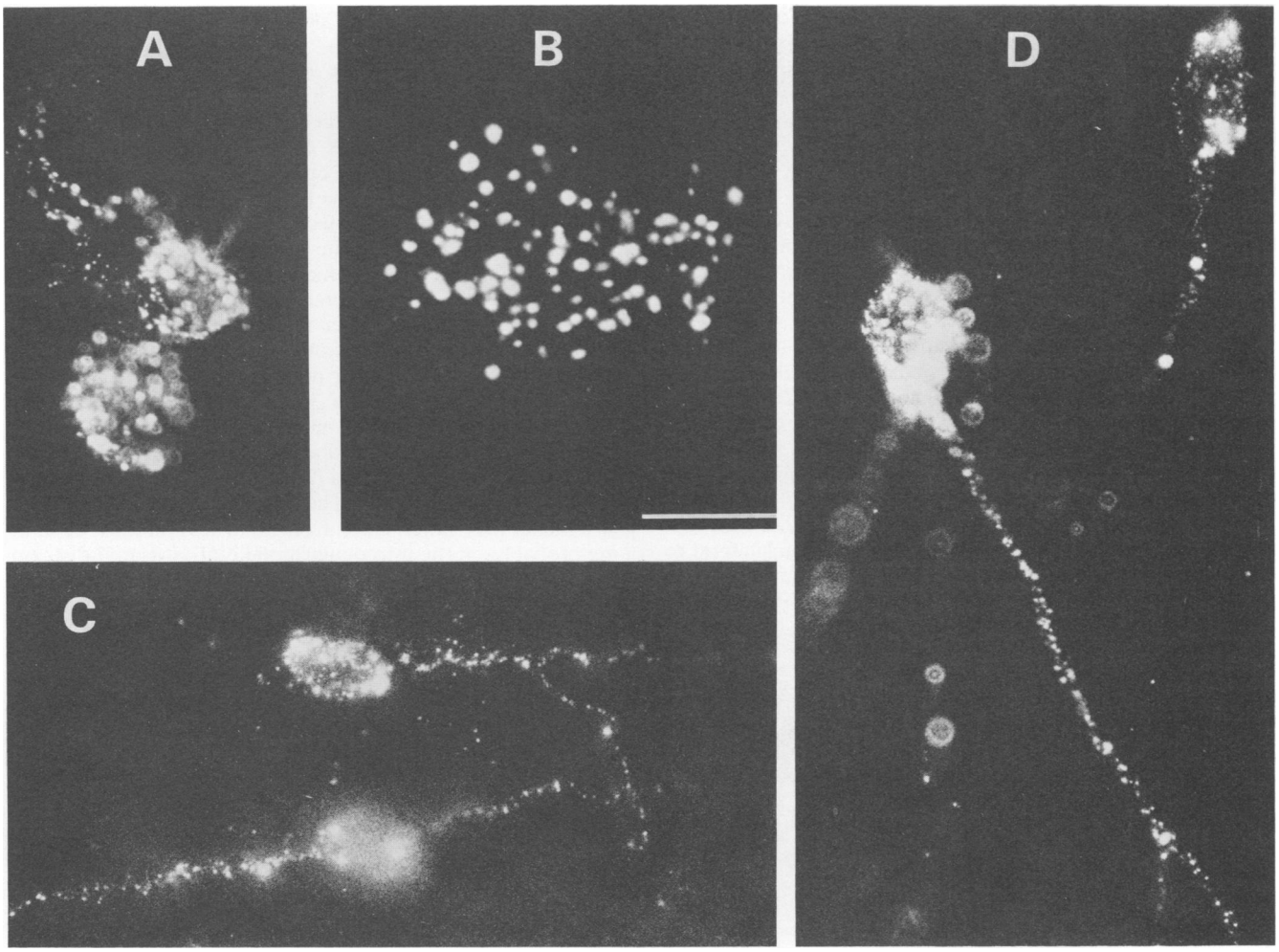

FIG. 2. Specific fluorescence in the neurons invaded from the eye: (A) 24 h after inoculation with CVS, virus present in cell bodies and axons in the ciliary ganglion; (B) 58 h after inoculation with Av01, a large infected neuron in the trigeminal ganglion; (C) 72 h, CVS, bipolar neurons in the medial pretectal area; (D) 72 h, CVS, one small and one larger multipolar (only one dendrite in focus) neuron in the EW. Bar, 20 μm.

loss of weight around days 6 to 7. The animals were sacrificed on days 1 to 5 (CVS) and 1, 2, 7, and 21 (Av01 and Av02) after inoculation. The eyes with the retrobulbar tissue containing the ciliary ganglion, the trigeminal ganglia, and the brains were rapidly removed and frozen. Serial 8- to 15-μm sections were made with a cryostat microtome. The sections, spread on slides, were fixed in acetone for 20 min and stored at −20°C. The presence of virus in this material was studied by the direct immunofluorescence technique, using fluorescein isothiocyanate-conjugated antinucleocapsid antibody obtained from rabbits, and purified and tested for specificity by the Federal Institute for Vaccines in Basel, Switzerland. Sections were rehydrated for 5 min in phosphate-buffered saline, covered with the antibody diluted 1:300 with phosphate-buffered saline, incubated for 45 min in a moist chamber at 37°C, rinsed three times for 5 min in phosphate-buffered saline, and mounted in a nonfluorescent mounting medium. The stained sections were observed at a magnification of ×625, using a Leitz Orthoplan microscope with the Ploemopak epiillumination system. The microscope stage with position transducers was interfaced to an X-Y plotter, allowing a precise mapping of fluorescent cells on drawings of brain sections, made at ×10 magnification, where the nuclei and tracts were outlined in a dark-field illumination. The drawings were compared with the atlases of rat brain (10, 12) to identify the infected structures. The entry of the viruses from the eye to neural elements and the subsequent intracerebral propagation of infection were studied by comparison of results obtained at different postinoculation intervals.

Sixteen animals inoculated with CVS and 13 animals

FIG. 1. Propagation of the parental (A) and mutant (B) rabies strains through the trigeminal (top), visual (center), and autonomic (bottom) interconnections between the eye and brain. Symbols: open arrows, direction of synaptic transmission; closed arrows, direction of propagation of the virus; circles, peripheral and central neuronal somata infected primarily (closed), secondarily (dots), and not infected (open) at each interval of time, indicated in hours after inoculation. Abbreviations: CG, ciliary ganglion; CGLd, lateral geniculated body (dorsal part); C8-Th1, spinal preganglionary sympathetic neurons; GG, trigeminal ganglion of Gasser; L, eye lens; NTOA, terminal nuclei of the accessory optic system; NST, terminal trigeminal sensory nucleus; R, retina; SCG, superior cervical sympathetic ganglion.

inoculated with the mutant strains have been considered in this study. About 60 sections per brain were studied. The sections covered all subdivisions of the rat brain. Electron micrographs from our previous studies were used for comparison with the results presented here.

RESULTS

Strain CVS. The intraocular injection of viruses seems to be a very efficient route of inoculation: only 1 of 96 animals studied so far was negative. All animals used in this study were infected. No fluorescence was found in control sections from noninfected material.

The invasion from the eye is characterized by four phases: (i) entry of the virus into the intraocular neural elements, (ii) ascendent propagation into the brain, (iii) intracerebral dissemination, and (iv) descendent propagation back to the eye. This is illustrated in Fig. 1A, which summarizes the nervous connections between the eye and the central nervous system.

At the periphery, specific fluorescence was detected first at 24 h after inoculation in the ciliary ganglion ipsilateral to the injected eye (Fig. 1A and 2A). Since the fluorescence in any type of permissive cells so far investigated can barely be detected before 16 to 18 h, this result indicates that the intraocular parasympathetic endings were the first neural target for the virus. At 48 h, the trigeminal ganglion and tractus also became fluorescent (Fig. 1A and 3). Thus, the second target for the virus entry into the brain was the intraocular terminals of the ophthalmic nerve.

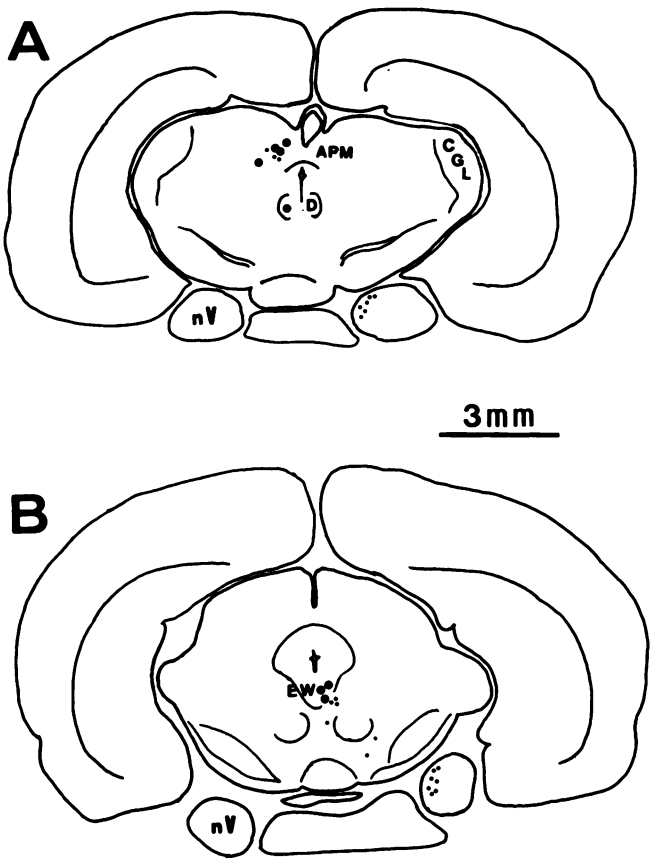

FIG. 3. Drawings of frontal sections through the diencephalomesencephalic region of the rat, 48 h after inoculation with CVS. The first infected neuronal somata (large dots) are found in the medial pretectal area, AMP (A), and in the parasympathetic oculomotor nucleus, EW (B). Fluorescent nerve fibers (small dots) are seen in the trigeminal tractus and oculomotor nerve roots ipsilateral to the infected eye (B). D, Nucleus of Darkschewitsch; nV, trigeminal nerve; CGL, lateral geniculated body (dorsal part).

In the brain, the virus was present 48 h after inoculation. It was constantly found in two small circumscribed regions. As expected, the ipsilateral mesencephalic Edinger-Westphal nucleus (EW), which sends the parasympathetic fibers to the eye, was infected (Fig. 1A, 2D, and 3B). The fluorescence was clearly seen not only in cell bodies but also in dendrites (Fig. 2D) and axons leaving the nucleus through the brain stem (oculomotor nerve roots; Fig. 3B). In addition, neurons of the area praetectalis medialis (APM), contralateral to the side of inoculation, were also intensely fluorescent (Fig. 1A, 2C, and 3A). As the APM neurons project directly to the retina (9), their endings were thus the third target for the virus. The number of neurons in these two nuclei was rather small, from 10 to several tens, depending probably on the intraocular uptake of virus and distribution of inoculum in the anterior and posterior eye chambers. In contrast to the EW and APM, neurons of the terminal trigeminal nuclei did not seem to be infected before 4 days. Thus the primary intracerebral foci of the CVS infection after intraocular inoculation seem to be exclusively the two small nuclei.

At 72 h after inoculation, the density of infected neurons in the EW and APM increased, but glial elements surrounding these neurons did not become fluorescent (Fig. 2C and D). From the two nuclei the virus propagated progressively through the brain, not per continuitatem (in which case the surroundings of the EW and APM would have been preferentially infected), but through functionally related, even distant neurons, as described earlier (7). In fact, the virus is an excellent tracer of neuronal connections, and, because of high convergence and divergence of nerve fibers, it infects practically all cerebral neurons. This happened at 96 h. At this time, the virus became detectable, in both eyes, in some retinal ganglionic cells, presumably belonging to the accessory optic system infected already at 72 h (see NTOA, Fig. 1A). Even at the terminal stage, the viruses seemed to remain in neuronal elements and to be transported in the axons (Fig. 4).

AvO strains. Twenty-four hours after inoculation, fluorescence was found in the anterior epithelium of the lens but not in the ciliary ganglion (Fig. 1B). At 48 to 58 h, very few neurons in the trigeminal ganglion became positive (Fig. 1B and 2B), and no fluorescence was detected in the brain. At day 7, when the animals presented a transient loss of weight, a few infected neurons were found, namely, in the cerebellum and basal ganglia. These neurons were probably invaded from the afferent trigeminal fibers as the other parts of brain did not show any fluorescence. Three weeks after inoculation, the brain again became negative and all remaining animals survived.

DISCUSSION

The intracerebral distribution of fluorescent neurons found in the later stages of infection was in agreement with results reported in a preliminary study of the intracerebral propagation of rabies virus from 72 to 96 h after inoculation (7). In this latter report, we suggested that the virus invades the brain via the accessory optic pathway. This interpreta-

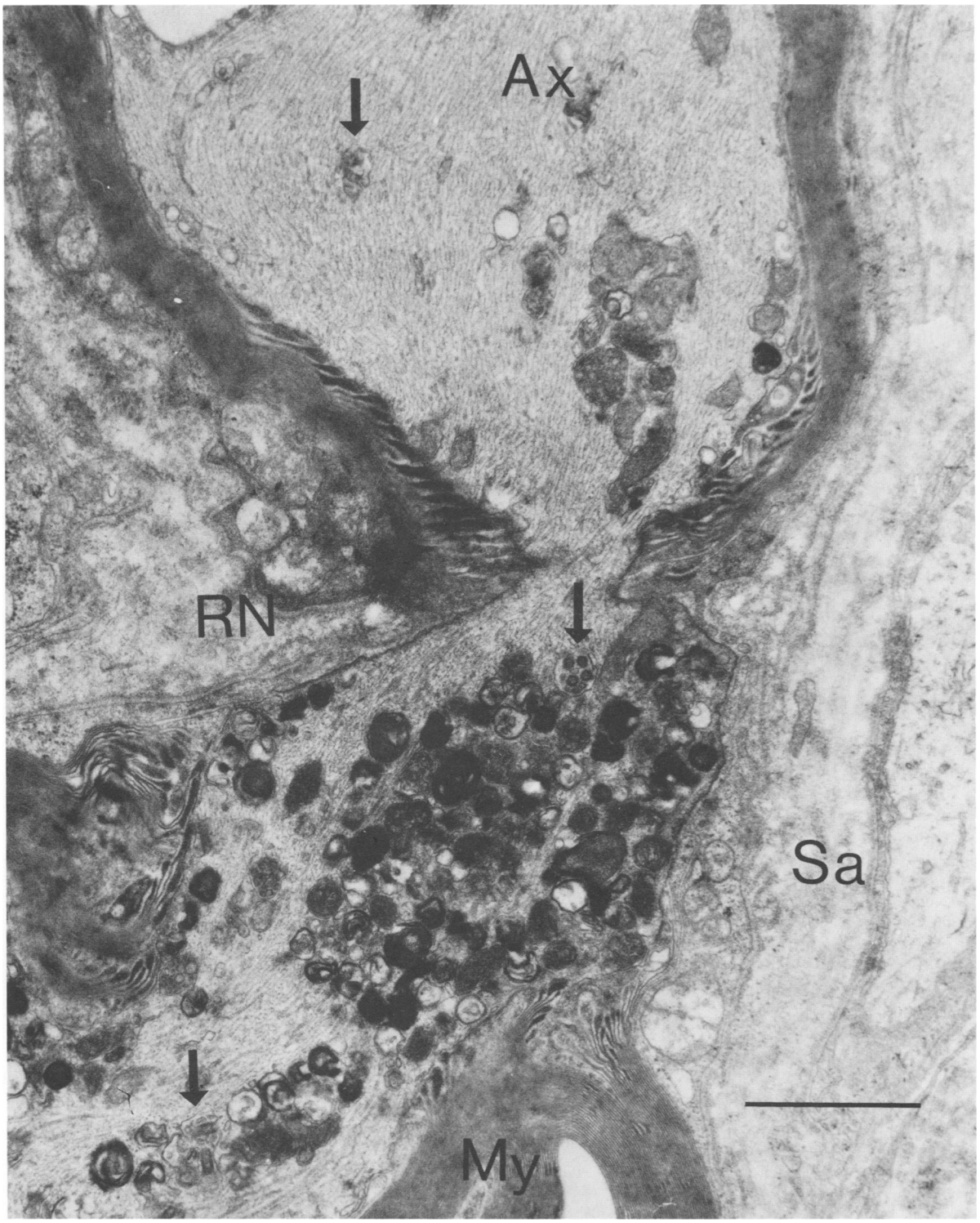

FIG. 4. Electron micrograph from the Gasserian ganglion at 120 h after inoculation. Arrows, CVS viruses transported within a myelinated axon (Ax). My, Myelin sheath; RN, Ranvier node with many abnormal inclusions, probably of viral origin. A satellite cell (Sa) surrounding the node is free of viruses. Bar, 1 μm.

tion of results obtained from already heavily infected brains cannot be held in view of the present data, obtained from the very early stages of infection. Instead, there are three routes of entry of the virus from the eye to the brain: the oculomotor parasympathetic nerve, the preopticoretinal pathway, and the ophthalmic nerve. Interestingly, the two remaining possible entries from the eye (Fig. 1), i.e., the retinal cells which project via the optic nerve to the lateral geniculate

body and the intraocular adrenergic terminals, were never primarily infected by either parental or mutant strains. Although part of the inoculum was constantly found in the posterior eye chamber, the retina and optic nerve did not become infected until very late and only secondarily from the brain. In agreement with previous reports (7, 15), the superior cervical sympathetic ganglion, innervated from the cervico-thoracic cord, was never infected within 5 days.

It has been proposed that the entry of rabies virus could be mediated by the nicotinic acetylcholine receptor (11, 16). Our results suggest a more complex situation. Among the intraocular endings infected by strain CVS, some are indeed cholinergic but of a muscarinic (and not nicotinic) type of transmission, thus involving a different receptor. In addition, strain CVS is able to penetrate the two other systems of nerve fibers (one efferent and one afferent). The nature of transmitters or membrane molecules in the pretectal fibers to retina or in the trigeminal nerve have not been determined so far. It could be speculated, of course, that these two systems might also be cholinergic. Alternatively, the virus could interact with other membrane components present only in some species of neurons or other cells. This seems to be the case for the mutants, which do not enter into the cholinergic terminals but which are still able to enter into the trigeminal sensory endings and which also penetrate the lens epithelium.

The primary intracerebral foci of rabies encephalitis after infection of CVS into the eye seem to be exclusively the EW and the medial preoptic nuclei (cf. Fig. 1A, 3A, and 3B) into which the virus is carried from the eye through the oculomotor and optic nerves at a speed of about 0.5 mm/h. The direction and speed of peripheral propagation, the direction of intracerebral propagation through defined neuronal pathways (e.g., the subfine appearance of virus in the retina), and the presence of virus within the axons even at day 5 all strongly support our hypothesis that the virus disseminates in the nervous system by retrograde axoplasmic flow (1).

The Av01 and Av02 glycoproteins differ from the CVS glycoprotein in a single amino acid. Our results show that this single substitution deeply modifies the host-virus interactions. Thus, the mutants lose their affinity for the intraocular parasympathetic and retinopetal fibers but become able to proliferate in the lens (cataract). They do invade the brain via the trigeminal nerve but the subsequent intracerebral dissemination is slow and abortive, probably because of intervention of the immune system.

ACKNOWLEDGMENTS

We thank U. Kihm from the Federal Institute for Vaccines in Basel for supplying the CVS virus and the antibody and D. Piguet for expert technical help.

This work has been supported by grant 3.318-0.82 from the Swiss National Science Foundation and by grants LA 040086 and LP 02431 from the Centre National de la Recherche Scientifique in France.

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