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Spread of the CVS Strain of Rabies Virus and of the Avirulent Mutant AvO1 along the Olfactory Pathways of the Mouse after Intranasal Inoculation

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After intranasal instillation in the mouse, rabies virus (CVS strain) selectively infected olfactory receptor cells. In the main olfactory bulb (MOB), infection was observed in periglomerular, tufted, and mitral cells and in interneurons located in the internal plexiform layer. Beyond the MOB, CVS spread into the brain along the olfactory pathways. This infection is specific to chains of functionally related neurons but at the death of the animal some nuclei remain uninfected. CVS also penetrated the trigeminal system. The avirulent mutant AvO1, carrying a mutation in position 333 of the glycoprotein, infected the olfactory epithelium and the trigeminal nerve as efficiently as CVS. During the second cycle of infection, the mutant was able to infect efficiently periglomerular cells in the MOB and neurons of the horizontal limb of the diagonal band, which indicates that maturation of infective particles is not affected in primarily infected neuronal cells. On the other hand, other neuronal cells permissive for CVS, such as mitral cells or the anterior olfactory nucleus, are completely free of infection with the mutant, indicating that restriction is related to the ability of AvO1 to penetrate several categories of neurons. From these observations, we concluded that CVS should be able to bind several different receptors to penetrate neurons, while the mutant would be unable to recognize some of them.

INTRODUCTION

Rabies is an enveloped RNA virus which causes encephalitis in all mammals and is considered a strictly neurotropic virus. Peripheral inoculation of the CVS strain of the virus results in a specific infection of the nervous system, especially of the neurons. Viral material has also been demonstrated in striated muscle at the site of injection of street rabies virus (Murphy and Bauer, 1974) and in various tissues of experimentally infected animals (Murphy *et al.*, 1973; Fekadu and Shaddock, 1986), including the salivary glands, the secretions of which are the major source of virus in animal vectors of rabies.

The only external protein of the virion is the glycoprotein (G), a transmembrane protein of 505 amino acids. The G protein plays a pivotal role in the pathogenicity of the virus because of its interaction with the host cells during the adsorption–penetration process and because of its function in the stimulation of the immune system. Few mutations which affect antigenic site II, the immunodominant antigenic site of the glycoprotein, slightly reduce the pathogenic power of the virus (Préhaud *et al.*, 1988). In antigenic site III, the other major antigenic site, substitution of the arginine in position 333 by an amino acid other than lysine completely abolishes the virulence of the virus for adult mice, whatever the dose and the route of inoculation (Coulon *et al.*, 1982a; 1983; Dietzschold *et al.*, 1983; Seif *et al.*, 1985; Tuffereau *et al.*, 1989).

These avirulent mutants are useful tools for studying the specificity of viral tropism for the central nervous system (CNS)[1]. AvO1, an antigenic mutant of the CVS strain, is currently the most studied mutant. Its mutation consists of a substitution of the arginine 333 by a glutamine (Seif *et al.*, 1985). Experiments in rats indicate that this avirulent mutant is restricted in infection of the CNS. Inoculation of CVS virus in the anterior chamber of the eye results in infection of both the trigeminal and the parasympathetic nerve ganglia and the retinopetal route, whereas AvO1 is only able to penetrate into the trigeminal nerve (Kucera *et al.*, 1985). A similar antigenic mutant called RV-194-2 has been isolated in another laboratory and was also used for experiments in animals (Dietzschold *et al.*, 1983).

[1] Abbreviations used: AON, anterior olfactory nucleus; CNS, central nervous system; GABA, Gamma aminobutyric acid; HDB, horizontal limb of the diagonal band; HRP, horseradish peroxidase; HSV1, herpes simplex type 1; IPL, internal plexiform layer; LC, locus coeruleus; LD50, lethal dose 50%; LPA, lateral preoptic area; MCPO, magnocellular preoptic nucleus; MHV, murine hepatitis virus; MOB, main olfactory bulb; PFU, plaque-forming unit; p.i., post-infection; SCG, superior cervical ganglion; VSV, vesicular stomatitis virus.

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The virulent and avirulent viruses have been compared in their ability to penetrate and propagate in the nervous system of the mouse along different pathways (Coulon *et al.*, 1989; Dietzschold *et al.*, 1985; Jackson, 1991). After injection in the forelimb both kinds of viruses infect the motor and sensory neurons with the same efficiency (Coulon *et al.*, 1989). After intramasseter inoculation, avirulent mutant was also found in both motor and sensory nuclei, while CVS was found only in the motor route (Jackson, 1991). In both cases, the block in the propagation of the avirulent mutant was observed as early as the second cycle of multiplication. This block would be the consequence of either abnormal maturation of the mutant in the neurons or nonrecognition of a specific cellular receptor by the glycoprotein of avirulent mutant. On the contrary, Dietzschold *et al.* (1985), who injected the virus intracerebrally, published that the two viruses spread similarly in the brain, although the infection by the avirulent mutant would progress slower than the CVS one.

In order to investigate more precisely the origin of the restricted multiplication of AvO1 in the CNS, we decided to follow the propagation of the virus in the olfactory pathways. Intranasal inoculation was used for several reasons. First, rabies virus is able to infect the CNS via the nasal route, since a few examples of airborne transmission of rabies have been described (Winkler, 1975); despite increasing interest for this route of infection, the mechanism of viral spread to the CNS has never been carefully examined. In addition, the olfactory system is anatomically well studied with several categories of connected neurons relatively easy to identify. Finally, the receptor cells of the olfactory epithelium are bipolar neurons whose dendrites are in contact with the external environment. Thus, these cells can be directly infected by a virus after it has been dropped in the nasal cavity without preliminary multiplication in nonneural tissues. Neuroreceptor axons terminate in the glomerular layer of the main olfactory bulb (MOB) where they synapse with dendrites of the periglomerular cells, the tufted cells, and the mitral cells (Halasz and Shepherd, 1983). The axons of neurons of the anterior olfactory nucleus (AON) (Switzer *et al.*, 1985), the horizontal limb of the diagonal band (HDB) (Záborsky *et al.*, 1986), and the dorsal raphe nuclei (DR) (McLean and Shipley, 1987) also terminate in the glomerular layer (Fig. 1). Since the progression of the infection seems to occur only through synapses (Coulon *et al.*, 1989), it should be possible to follow at least two successive cycles of multiplication after instillation of the virus in the nose. Mice were infected by this route with CVS or AvO1, and the spread of the viruses in the CNS was followed with immunohistochemical techniques.

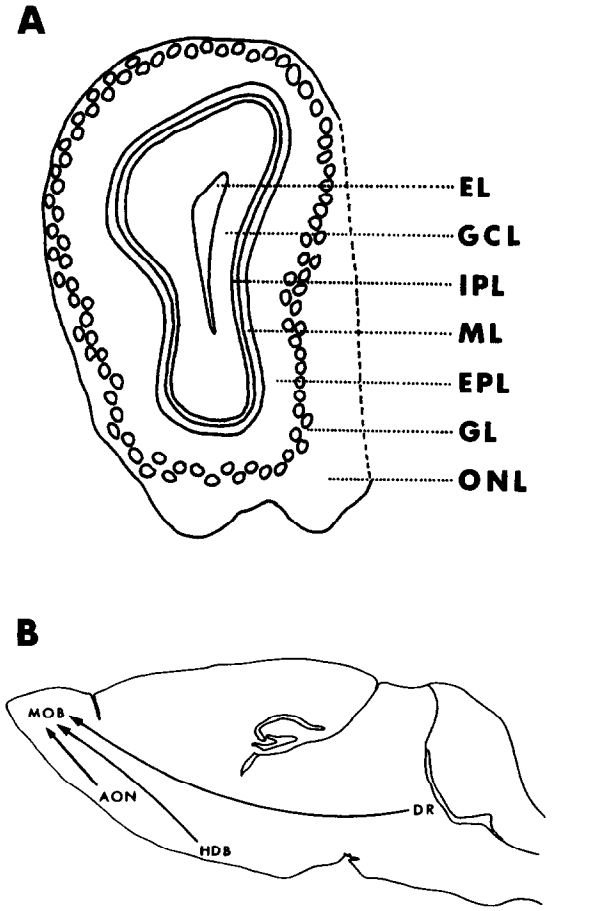

FIG. 1. Anatomy of the olfactory system. (A) Frontal section of the main olfactory bulb (MOB). Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; ML, mitral layer; IPL, internal plexiform layer; GCL, granular cell layer; EL, ependymal layer. (B) Longitudinal section of the brain with the localization of the neurons projecting in the glomerular layer of the main olfactory bulb. Abbreviations: AON, anterior olfactory nucleus; HDB, horizontal limb of the diagonal band; DR, dorsal Raphe nucleus.

MATERIALS AND METHODS

Viruses

The CVS strain of rabies and its avirulent derivative AvO1 were grown in BHK-21 cells. Concentration of the viruses from cell culture supernatants was performed as described by Coulon *et al.* (1989).

Experimental procedures

Six-week-old female OF1 random bred mice (IFFA-CREDO, St Germain sur l'Arbresle, France) were anesthetized with 100 μl of Equithesin (4% chloral hydrate, 16% pentobarbitol). Three microliters of concentrated CVS or AvO1 viruses in TD (Tris–HCl, pH 7.4; NaCl, 150 m*M*; KCl, 5 m*M*; Na_2HPO_4, 0.7 m*M*) + 1 m*M* EDTA were instilled in the right nostril using a Hamilton

syringe connected to a stretched catheter. The mice were maintained on their backs for 15 min after instillation.

At various times after infection the animals were successively perfused intracardially with PBS (phosphate-buffered saline) (NaCl, 150 m*M*; Na_2HPO_4, 7.4 m*M*; KH_2PO_4, 2.4 m*M*) (20 ml), 2% paraformaldehyde in PBS (150 ml), and 10% sucrose in PBS (50 ml) using a peristaltic pump. After dissection, the brain was kept in PBS + 10% sucrose for 24 hr for cryoprotection; the nose was decalcified in PBS + 0.1 *M* EDTA for 5 days and then kept for 24 hr in PBS + 10% sucrose at 4°. The tissues were frozen at −70°.

Serial 30-μm frontal sections of the entire brains were made in a cryostat microtome (Bright, Huntingdon, England). An average of 500 sections per brain was obtained. All the sections were treated for immunofluorescence and the total number of positive neurons was determined for each infected animal. In a few instances, sections were collected on three alternate series of gelatin-coated slides. A first series was treated for immunofluorescence, a second for immunoperoxidase, and a third was kept in reserve. Several noses were also cut in totality and all sections were treated for immunofluorescence.

Immunofluorescence

The sections were permeabilized in PBS + 1% Triton X-100 for 30 min at room temperature, washed three times in PBS, and then treated with fluorescein isothiocyanate-conjugated anti-nucleocapsid antibodies (Pasteur Production). After washing with tap water, the slides were mounted in Elvanol (Tris-PO_4, 50 m*M*; polyvinyl alcohol, 20%; glycerol, 20%; pH 8.2) and then individually examined using a uv microscope.

Immunoperoxidase

The sections were permeabilized in PBS + 1% Triton X-100 for 30 min and then washed three times with ELISA III buffer (NaCl, 150 m*M*; Tris–HCl, 50 m*M*; EDTA, 1 m*M*; Tween-20, 0.05%; bovine serum albumin, 0.1%; pH 7.4). After a 3-min incubation in 0.3% H_2O_2 and three washes in ELISA III, the tissues were incubated for 24 hr at 4° with a rabbit anti-nucleocapsid antibody (1/300 in ELISA III). The sections were washed three times in ELISA III and incubated for 1 hr at 37° with an anti-rabbit biotinylated antibody (ABC kit "Elite," Vector, Burlingame, CA) diluted 1/200 in ELISA III. The signal was amplified by incubation (20 min at 37°) in a mixture of avidin and biotinylated peroxidase. After washing, the sections were incubated in a substrate of diaminobenzidine (Sigma, St Louis, MO) at 1 mg/ml in Tris–HCl, 0.1 *M*, pH 7.4, plus an equal volume of 0.02% H_2O_2 in water. The reaction was stopped after discarding the mixture and washing with tap water. Tissues were counterstained with Giemsa. After washing and drying, the sections were mounted with DPX (BDH, Poole, England). Identification of the cerebral structures was performed according to the stereotaxic atlas of the mouse brain by Lehmann (1974) and that of the rat brain by Paxinos and Watson (1986).

RESULTS

Pathogenic powers of CVS and AvO1 viruses via the olfactory route

Under our experimental conditions the olfactory route is an efficient route of infection for the CVS strain. The median lethal dose (LD50) was determined and corresponded to 50 PFU. By comparison, the intracerebral LD50 is 1 PFU, the intramasseter LD50 at least 10^3 PFU (Coulon *et al.*, 1982b), and the LD50 after injection in the forelimb about 5×10^4 PFU (Coulon *et al.*, 1989). As expected, the AvO1 mutant was completely avirulent by the olfactory route even when 7.5×10^7 PFU of virus was instilled.

Penetration of CVS and AvO1 viruses in the olfactory epithelium

Undiluted cell culture supernatant containing CVS (5×10^4 PFU or about 1000 intranasal LD50) was used in preliminary experiments. The first round of multiplication in the nasal cavity occurred only in the olfactory receptor cells. Twenty-four hours after instillation few neurons (around 25 in the whole organ) contained enough material to be detectable by immunofluorescence. At 2 days postinfection, this number increased to reach a mean value of 150 and remained relatively stable during the following 2 days. The infected neurons were usually not adjacent (Fig. 2B).

In order to increase the number of infected receptor cells, we instilled concentrated virus. Following instillation of 4×10^7 PFU of CVS (8×10^5 in LD50), the number of positive neurons reached 2 to 5×10^3 at 2 days postinfection (p.i.), which represents less than 0.1% of the total number of olfactory receptor cells. The infected cells were consistently found mainly in the bottom of the turbinates (Fig. 2A) and in the septal organ. Complementary experiments using horseradish peroxidase (HRP) instead of virus showed that the epithelial areas where all cells were HRP-positive were also the infected ones (data not shown). Since only a minority of the olfactory receptor cells were infected, it is not known if all the receptor cells are permissive for rabies. Infected neurons were generally found in the

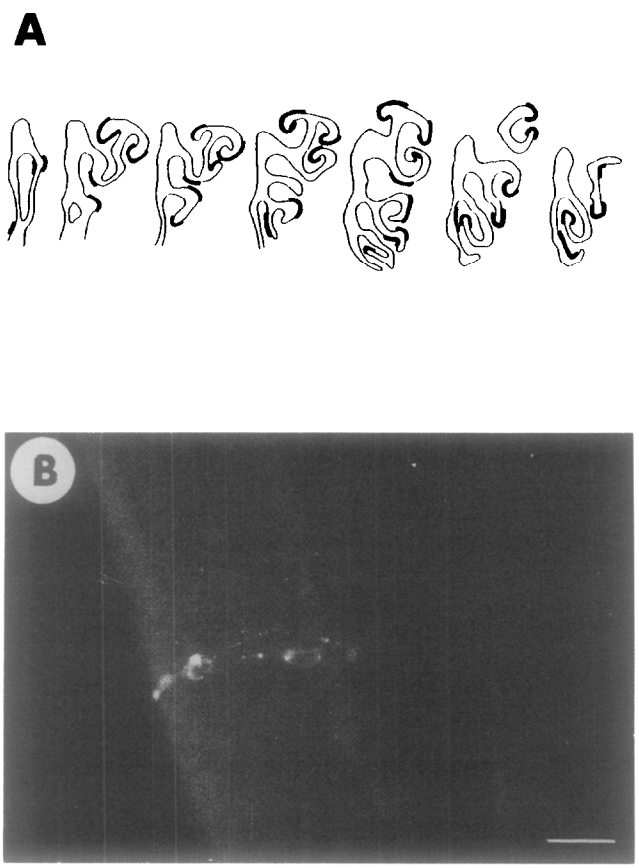

FIG. 2. (A) Localization of the infected olfactory receptor cells in the nasal cavity following instillation of CVS and AvO1 viruses in the right nostril. The distribution of the labeling is represented by heavy lines. (B) CVS-infected olfactory receptor cells identified by immunofluorescence. Bar = 25 μm.

right olfactory epithelium since the virus was instilled into the right nostril. In a few mice (about 1 of 10), some infected cells were also detected on the left nasal fossa, which was not unexpected since the fossae communicate.

The avirulent mutant AvO1 showed a comparable capability to infect receptor cells. The ratio between infected cells and PFU is the same for both viruses and the infected cells were found in the same epithelial areas (data not shown).

At Day 6 or 7 when CVS-infected mice die, olfactory receptor cells were still infected, but their number decreased. In the AvO1-infected mice which survived the infection, the olfactory epithelium was clear of virus after Day 7. The vomeronasal organ and the respiratory epithelium never appeared to be infected, even at the latest stage of the infection.

Infection of the main olfactory bulb after instillation of CVS

The first neurons detected as infected by immunofluorescence in the MOB were observed 48 hr after instillation. This agrees with the occurrence of a first cycle of infection in the olfactory epithelium before reaching the bulb. At 2 and 2.5 days p.i. only a few cells located in the glomerular layer appeared infected in the ipsilateral MOB (Table 1). At Day 3, the number of infected periglomerular and/or external tufted cells significatively increased. As these cellular types were difficult to distinguish (Fig. 3A) they were counted together (Table 1). A spotted fluorescence was observed in some glomeruli even in the absence of surrounding infected cells, suggesting the presence of viral nucleocapsids in the axonal endings of receptor cells. Mitral cells and large size neurons located in the internal plexiform layer (IPL) also appeared infected (Fig. 3B). These cellular elements were also counted together (Table 1). The use of the immunoperoxidase method allowed us to estimate that the mitral cells and IPL neurons were equally represented. Few positive medium size neurons were found in the external part of the granular layer. No labeling was noted in the contralateral bulb, except when both fossae were primarily infected. From Day 4 the contralateral MOB became infected. In some animals the number of infected mitral cells could be higher in the contralateral bulb than in the ipsilateral one (Table 1). At Day 5 nearly all the mitral cells in the contralateral bulb appeared infected (Fig. 3C). From this time to death it was not possible to count the infected cells because they were too numerous. Nevertheless, very few cells in the granular layer contained viral nucleocapsids (Fig. 3C).

Infection of the main olfactory bulb after inoculation of AvO1 mutant

At Days 3 and 4 neurons appeared infected in the periglomerular region of the ipsilateral bulb, but their number was apparently reduced compared to that noted in the CVS infection at the same time. As for CVS infection, all the infected cells in the glomerular layer were counted together (Table 2). Accumulation of nucleocapsids in the glomeruli was also detectable (Fig. 3D). Almost no labeling was observed in the mitral cells and the neurons located in the IPL. Among 11 animals observed at Day 3 or 4 p.i., only a single infected mitral cell was detected in each of two animals. Beyond Day 5 p.i., few mitral cells were detectable in almost all observed animals (Table 2). These infected cells were found in the ipsi- or the contralateral bulb and their number remained low and did not account for more than 0.1% of the mitral cells [assuming a total number of mitral cells around 50,000 as estimated in the rat (Meisami and Safari, 1981)].

Other structures of the brain early infected with CVS

At Day 2 or 2.5 p.i., very few infected neurons were detected in two nuclei known to project into the glo-

TABLE 1

INFECTION BY CVS OF THE BULBAR NEURONS AND THE BRAIN STRUCTURES DIRECTLY CONNECTED TO THE GLOMERULI

	MOB								
	Periglomerular + tufted		Mitral + internal plexiform		AON		HDB		
Days	L	R	L	R	L	R	L	R	Raphe
2	0	6	0	0	0	0	0	3	0
2.5	0	7	0	1	1	3	0	22	0
	0	7	0	0	0	1	0	2	0
3	1	81	0	67	50	600	0	376	0
	13	32	14	49	31	330	14	385	0
	0	41	0	12	16	59	0	150	0
	0	56	0	33	0	43	0	100	0
	0	116	0	92	168	664	48	880	0
	0*	135	0	117	81	405	15	852	0
	0*	21	0	34	3	18	0	72	0
	0*	6	0	6	0	15	0	135	0
4	1250	1350	2000	900	+++	++	++	++	∓
	1500	1500	2000	1500	+++	+++	∓	+++	∓
	123*	105	0	45	++	+++	++	+++	∓
	963*	747	675	1638	+++	+++	+	++	∓

Note. Mice were instillated with 4×10^7 PFU of CVS virus and sacrificed at various times after infection. The brains were processed as described under Materials and Methods. Each line represents one infected animal in which the total number of infected neurons was determined.

* Only one of three sections was treated for immunofluorescence; the number of labeled cells obtained was tripled for the comparison with the other series. AON, anterior olfactory nucleus. HDB, horizontal limb of the diagonal band. MOB, main olfactory bulb.

merular layer: the AON and the HDB. In one animal sacrificed 2.5 days after instillation, 3 of the 4 infected cells found in the AON were located in the ipsilateral nucleus and the other in the contralateral one. At Day 3 p.i., the AON was found to be consistently infected ipsilaterally and to a lesser extent contralaterally (Fig. 4). It is not surprising to find infected neurons in the contralateral AON, because the AON is an important contralateral source of MOB afferents (Carson, 1984). There were between 18 and 664 infected cells in the right AON (Table 1); between 0 and 168 infected neurons were detected in the left AON. Like the AON, the ipsilateral HDB was consistently infected at Day 3 p.i. (Fig. 5A). In some animals (three of seven), labeled cells were observed in the contralateral HDB (Table 1). In the first animal the contralateral labeling of HDB was the consequence of a bilateral infection of the olfactory epithelium, since the contralateral MOB was infected. This was not the case for the other two animals; given that, unlike the AON, HDB does not project contralaterally in the MOB (Carson, 1984), infection in these animals was due to a third cycle of multiplication. In these animals, some third order labeled cells were also found in the piriform cortex (Fig. 5A). Other regions of the brain were consistently found to be infected ipsilaterally at Day 3 in addition to HDB and AON. They were the lateral preoptic area (LPA) and the magnocellular preoptic nucleus (MCPO) (Fig. 5A).

Early infection of the brain during the AvO1 infection

A very different situation was found in AvO1-infected animals. At Day 3 or 4 p.i., HDB appeared as efficiently infected with the mutant as with the wild type, while there was no infection in the AON (Table 2). The MCPO which was labeled with CVS was also permissive for the AvO1 mutant (Fig. 5B), while the LPA showed no labeled cells. In the AON few cells were labeled from the fourth day of infection, and in any case the number of infected cells remained very low compared to the wild type (less than 0.1%).

Later infection of the brain with CVS and AvO1

The invasion of the brain by CVS after the third day of infection involved many nuclei which were not necessarily related to the olfactory pathways. A precise description will be published elsewhere; in this paper only some relevant points allowing a better understanding

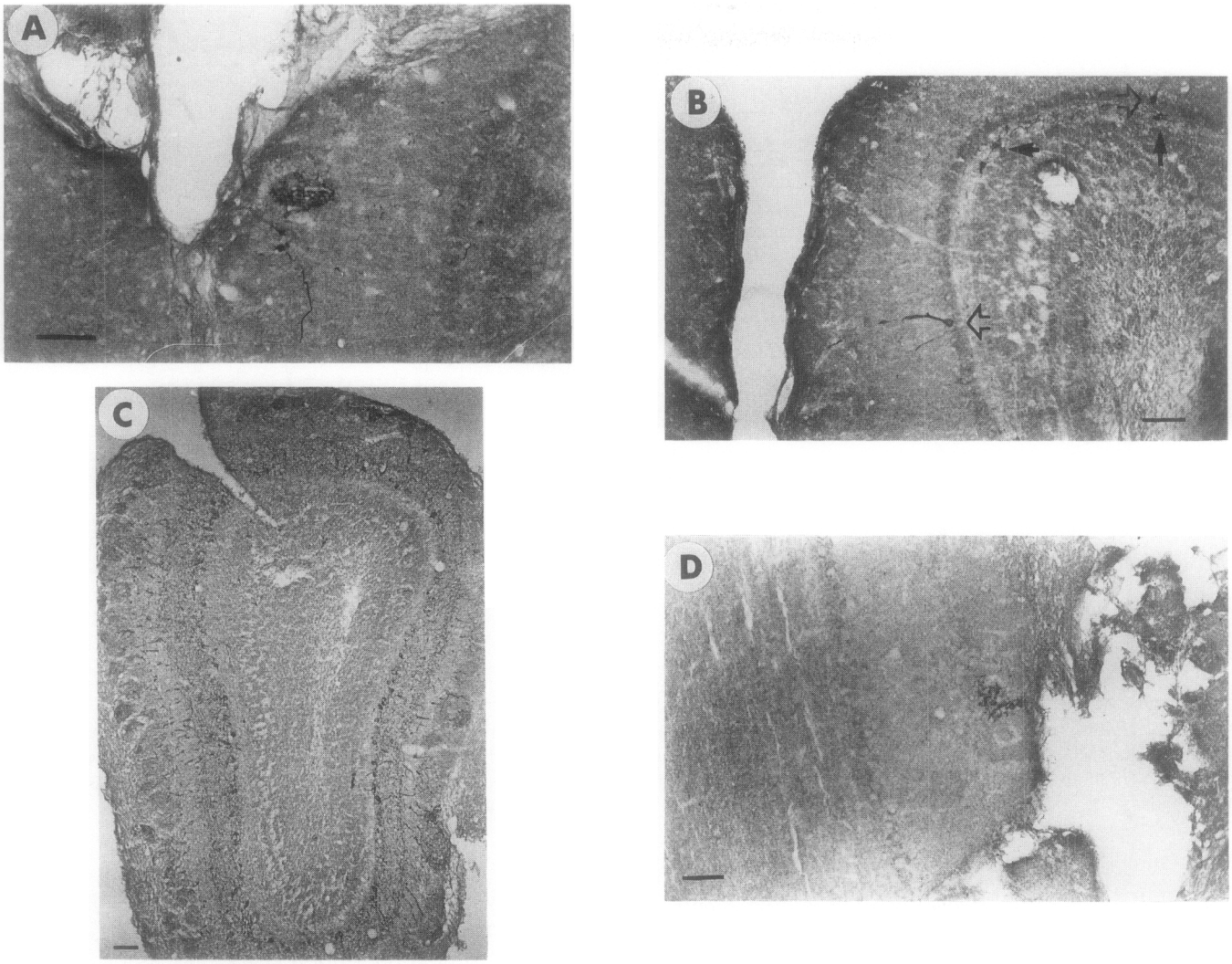

FIG. 3. Detection of viral antigens by immunoperoxidase in the MOB after infection with CVS (A to C) or AvO1 (D). (A) Labeled glomeruli surrounded by infected periglomerular and/or external tufted cells at Day 3 p.i. (B) Rabies virus antigens in mitral cells (open arrows) and neurons of the IPL (solid arrows) at Day 3 p.i. (C) CVS-infected mitral cells in the contralateral bulb at Day 5 p.i. Note the presence of few infected neurons in the granular cell layer. (D) Glomerulus infected by AvO1 at Day 4 p.i. Bar = 100 μm.

of mechanisms which could explain the difference in pathogenicity between CVS and the AvO1 mutant will be described.

Several animals were examined at each day of infection, in order to distinguish the consistently infected areas from those rarely infected. Two different situations were observed. (i) The same structures were infected by both strains, even if fewer neurons were often involved for the mutant strain. Nuclei with at least 20% infection compared to the wild type were considered to be in this category. Infection with the mutant could be observed at nearly the same time as for the wild type, like in the orbital cortex and in the amygdala. For most nuclei infected later, the appearance of antigen with the mutant was slightly delayed (up to 1 day). This was the case for the Purkinje cells in the cerebellum and the ventromedial thalamic nucleus. (ii) Some structures were infected with the wild type and not, or scarcely, infected with the mutant. This was the case for two nuclei related to the olfactory pathways, the septal area (one of the most heavily infected areas for the wild type) and the raphe nucleus. Another example is the hippocampus which was infected by the wild type as early as Day 4 p.i. [mainly pyramidal cells (Fig. 6)] and completely invaded by Day 5. With the mutant strain only two of five animals showed a small focus of infected cells in this structure at Day 5. Of three animals sacrificed at Day 6 or 7, two were free of infection in the hippocampus, while the latter showed 10 infected cells. Moreover, in these small infected areas very few pyramidal cells were concerned, most of the infected cells being in the stratum lacunosum molecu-

TABLE 2

INFECTION BY AVO1 OF THE BULBAR NEURONS AND THE BRAIN STRUCTURES DIRECTLY CONNECTED TO THE GLOMERULAR LAYER

	MOB								
	Periglomerular + tufted		Mitral + internal plexiform		AON		HDB		
Days	L	R	L	R	L	R	L	R	Raphe
3	0	23	0	0	0	0	0	76	0
	0	23	0	0	0	0	0	110	0
	0*	105	0	0	0	0	0	200	0
	20	40	0	0	0	0	16	180	0
	0*	12	0	0	0	0	0	9	0
	0*	12	0	0	0	0	0	192	0
4	0	17	0	1	0	11	0	295	0
	0*	12	0	0	0	0	0	306	0
	0	96	0	1	2	27	0	±	0
	0	4	0	0	0	24	0	384	0
	0*	33	0	0	0	0	0	264	0
5	3*	48	0	12	nd	nd	nd	nd	nd
	11	nd	1	nd	14	18	50	84	18
	0	19	0	0	33	23	19	214	26
	0	15	0	3	16	9	90	510	nd
6	42*	18	45	12	3	0	++	++	nd

Note. Mice were instillated with 7.5×10^7 PFU of AvO1 virus and sacrificed at various times after infection. The brains were processed as described under Materials and Methods. Each line represents one infected animal in which the total number of infected neurons was determined.

* Only one of three sections was treated for immunofluorescence; the number of labeled cells obtained was tripled for the comparison with the other series. AON, anterior olfactory nucleus. HBD, horizontal limb of the diagonal band. MOB, main olfactory bulb. nd, not done.

lare. Among the other nuclei known to project directly to the MOB, the locus coeruleus was never infected either by the wild type or by the AvO1 mutant.

Infection of the trigeminal nerve

In the course of our study, we observed that the trigeminal nerve was consistently infected even at the early stage of the infection with CVS. Systematic observations of the Gasser ganglions in immunofluorescence showed that this structure was infected at Day 2 (data not shown). This indicates that the virus was able to penetrate the trigeminal endings without injury to the nasal cavity. The AvO1 mutant directly infected the trigeminal nerve in the same manner as CVS (data not shown). At Day 4, infected neurons were detected in the mesencephalic nucleus of the trigeminal nerve in both infections (Fig. 7), but the number of labeled cells was lower in the AvO1 than in the CVS infection.

DISCUSSION

Several neurovirulent viruses invade the brain along olfactory pathways: vesicular stomatitis virus (VSV) (Lundh *et al.*, 1987, 1988), murine hepatitis virus (MHV)-JHM (Barthold, 1988; Perlman *et al.*, 1989), and herpes simplex type 1 (HSV1) (McLean *et al.*, 1989). The rabies virus, under appropriate conditions, is also able to infect the CNS of various mammals after instillation in the nose, but the mechanism of centripetal spread of the virus to the CNS has never been clarified. Here we provide a careful description of the first cycles of viral infection, including timing and identification of permissive neurones. We also precisely compared the CVS (pathogenic) and AvO1 (avirulent) viruses for their ability to invade the CNS of the mouse after nasal instillation. Both viruses infect the olfactory epithelium but not the respiratory epithelium. The same specificity was previously reported for VSV, another rhabdovirus (Lundh *et al.*, 1987). This is not the case for MHV-JHM infection in which the respiratory and olfactory epithelia are both infected (Barthold, 1988).

A second route of infection is used by CVS and the avirulent mutant after nasal instillation. It is the trigeminal route which is followed by other neurotropic viruses like MHV-JHM (Perlman *et al.*, 1989) and HSV1 (McLean *et al.*, 1989). As early as 2 days postinfection viral material was revealed by immunofluorescence in the Gasser ganglion. This result confirms previous ex-

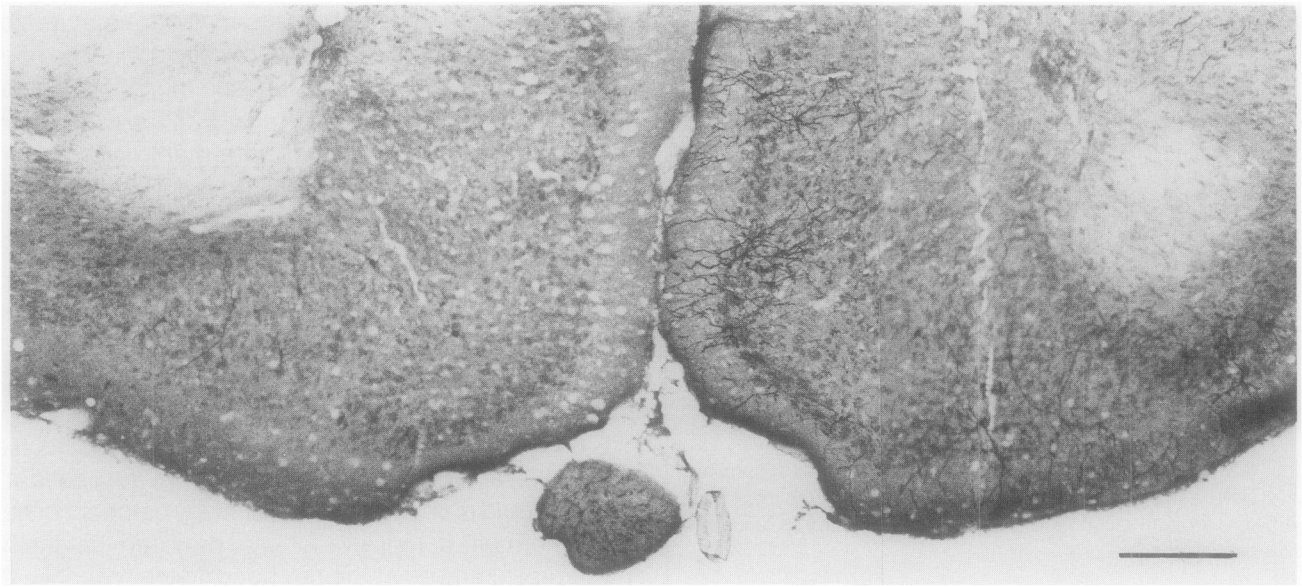

FIG. 4. Infection of the AON 4 days after instillation of CVS. Detection by immunoperoxidase. Bar = 200 μm.

periments in which CVS and AvO1 viruses had been shown to infect the trigeminal ganglion after intraocular inoculation (Kucera *et al.*, 1985). It also suggests that trigeminal endings are accessible to the virus without injury of the nasal fossae.

Very few infected neurons are detectable 24 hr p.i. in the olfactory epithelium, although the distance between the dendrite endings and the cell body is very short. The increase in the number of infected neuroreceptors observed between Days 1 and 2 cannot be due to a second cycle of infection since infected cells are usually not adjacent. The timing of the first cycle of multiplication in the olfactory epithelium can thus be estimated at 48 hr. This is longer than in cell cultures or in other neurons. For example, after injection in the forelimb of the mouse, viral material can be detected in the spinal cord and the dorsal root ganglia as early as 18 hr p.i. (Coulon *et al.*, 1989). The longer cycle observed after intranasal exposure could be due to a lower temperature in the nasal cavity compared to that of the body or to a trapping of the virus during the passage across the mucus layer covering the epithelium.

As already mentioned, we did not observe patches of infected neurons in the olfactory epithelium. This indicates that neurons adjacent to the primary infected cells could not be infected, either because there is no budding of virions at the cell body level or because

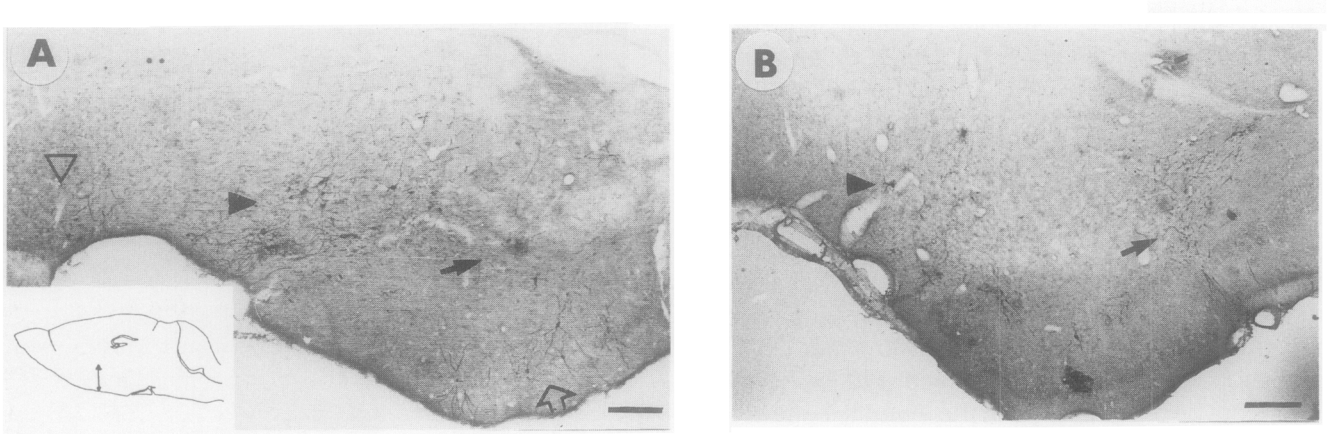

FIG. 5. Secondary infection of the HDB region after intranasal instillation of CVS (A) or AvO1 (B). (A) Viral antigens in the HDB (solid arrowhead), the LPA (open arrowhead), and the MCPO (solid arrow) at Day 3 p.i. with CVS. Note some pyramidal neurons infected in the piriform cortex (open arrow). Detection by immunoperoxidase. (B) Viral antigens in the HDB (solid arrowhead) and the MCPO (solid arrow) at Day 4 p.i. with AvO1. Detection by immunoperoxidase. Bar = 200 μm.

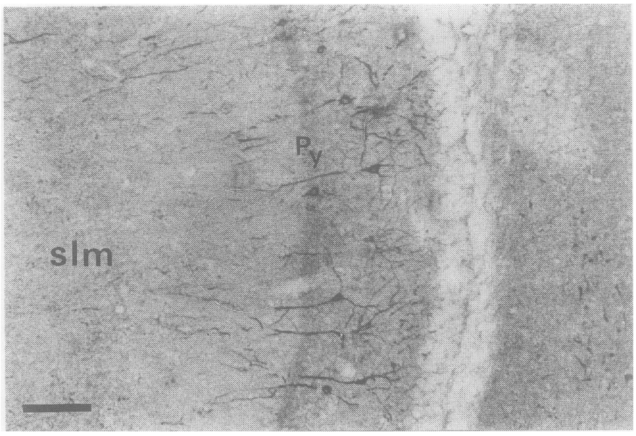

FIG. 6. Rabies virus antigens in pyramidal cells of the hippocampus 4 days after instillation of CVS. Abbreviations: Py, pyramidal layer; Slm, stratum lacunosum moleculare. Detection by immunoperoxidase. Bar = 100 μm.

there is no receptor for these virions at this level. A comparable observation was reported in dorsal root ganglia infected with CVS (Coulon *et al.,* 1989). These observations argue for strictly transsynaptic transmission of rabies virus. Rabies virus is different in this respect from VSV, which has been shown to bud at the level of cell bodies of the olfactory receptor cells (Lundh *et al.,* 1987), and from HSV1, which gives patchy label at least in the olfactory epithelium and the bulb, thus suggesting a transmission from cell body to cell body in addition to the transsynaptic transport (McLean *et al.,* 1989).

At the third day of infection with CVS, the three categories of bulbar neurons known to have dendritic terminals in the glomerular layer are infected. These are the periglomerular, tufted, and mitral cells (Halasz and Shepherd, 1983). Another category of cells which was found to be consistently infected in the IPL anatomically resembles the horizontal cells described by Schneider and Macrides (1978). But, this latter category is not currently thought to make connections with olfactory neuroreceptors. These neuroanatomical connections identified with rabies virus used as a tracer will be described in more detail elsewhere. Among the nuclei known to project into the glomerular layer, the AON and the HDB are infected by the wild type. All these neurons are secondarily infected with virions produced by the olfactory receptor cells. By comparison, in the MOB, AvO1 is only able to infect cells located around the glomeruli, the mitral and the horizontal cells not being infected with the mutant. In the brain, the AvO1 mutant is able to infect secondarily the HDB. In contrast the AON is not permissive for the mutant. These results allow us to conclude that AvO1 is able to replicate (because AvO1-infected neurons are detectable by immunohistochemistry) and to mature (because of the infection of synaptically connected neurons) in the olfactory epithelium and the trigeminal ganglion. Unlike Dietzschold *et al.* (1985), we concluded that the spread of the avirulent mutant does not proceed at a slower rate than the spread of CVS but rather implied less categories of neurons.

Why are certain neurons not permissive for AvO1? Either the mutant is unable to replicate normally in these cells or it does not bind to a specific receptor(s) at the synaptic junctions of these cells. The first hypothesis seems unlikely because the mutation of AvO1 affects the glycoprotein, and this protein is not known to be involved in the replication of rhabdoviruses (see Wertz *et al.,* 1987, for a review). The replication of AvO1 could probably take place in any category of neurons normally permissive for CVS provided that the nucleocapsid arrives at the cell body. More probably, the restricted tropism of AvO1 is related to the lack of recognition of receptors present at synapses of different groups of neurons. The strong tropism for neuronal cells suggests that viral receptors should be specific to neurons and could be a protein. The suggestion that the acetylcholine receptor could be the receptor for rabies virus has been made on the basis of competitive fixation experiments between rabies virus and α-bungarotoxin on cultured myotubes (Lentz *et al.,* 1982), and sequence homology between the 189–214 region of the rabies glycoprotein and the 30–56 region of the long neurotoxins (Lentz *et al.,* 1984). Since AvO1 is able to penetrate several categories of neurons, and is restricted for others, the wild type should be able to bind to several different receptors, and thus the acetylcholine receptor cannot be the unique receptor for rabies. Some of our results argue in favor of the acetyl-

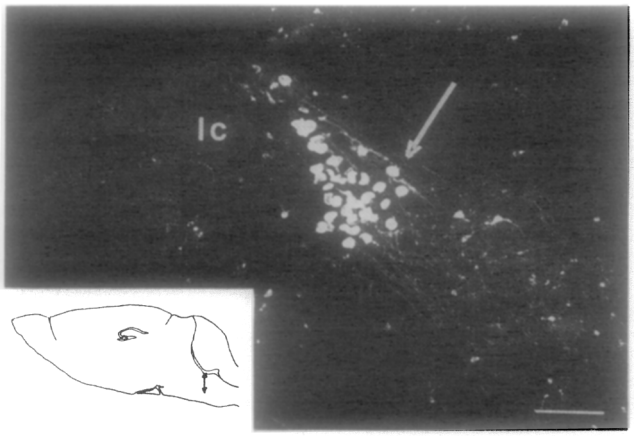

FIG. 7. Accumulation of viral nucleocapsids in the mesencephalic nucleus of the trigeminal nerve (arrow) 4 days after instillation of CVS. Note the absence of labeling in the locus coeruleus (lc). Detection by immunofluorescence. Bar = 200 μm.

choline receptor hypothesis: indeed, neurons of the HDB ending in the glomerular layer of MOB are cholinergic (Záborszky *et al.*, 1986), and it is likely that the horizontal neurons of the IPL are the cholinoceptive neurons described by Nickell and Shipley (1988). On the contrary, other permissive cells respond to various neurotransmitters: the periglomerular cells to GABA, dopamine, and enkephaline; the tufted cells to glutamate/aspartate, dopamine, and substance P; and the mitral cells to glutamate/aspartate (Halasz and Shepherd, 1983). Of course it is also possible that other molecules which are not implicated in the binding of neurotransmitters are used by rabies virus as receptors.

Interestingly, even during CVS infection, some categories of neurons are not infected by the virus. This is the case for the granular cells in the MOB and the locus coeruleus (LC), a nucleus directly connected to the MOB (Shipley *et al.*, 1985). These cells are secondarily (or later) infected in rats instilled with HSV1 (McLean *et al.*, 1989) or in mice instilled with VSV (Lundh *et al.*, 1988). If, as suggested by Shipley (1985), the LC is connected to the granular cells in the MOB, it is not surprising that this nucleus was not infected early since granular cells are not permissive for the CVS strain of rabies. Nevertheless the absence of infection of the LC even when the mouse was moribund suggests that these neurons are nonpermissive to the virus. The LC has a noradrenergic projection in the MOB (Shipley *et al.*, 1985). Another group of neurons responding to noradrenaline, the superior cervical ganglion (SCG), does not seem permissive for the rabies virus. While injection of pseudorabies virus (Herpesvirus) in the anterior chamber of the eye of rats results in infection of the SCG (Dolivo *et al.*, 1981), the injection of rabies virus at the same site does not result in the infection of this ganglion (Tsiang *et al.*, 1983; Kucera *et al.*, 1985).

It has long been stated that the rabies virus is transported from the synapses to the cell bodies by retrograde axonal flow. Our experiments confirm our previous results that the virus can travel via anterograde as well as retrograde flow. Indeed, during the first cycle of infection the virus is transported in an anterograde fashion in the olfactory epithelium and in the trigeminal nerve. During the second cycle, the virus uses the two types of transport: (i) Anterograde in the mitral and the periglomerular cells in the MOB and (ii) retrograde in the horizontal cells of the MOB and the neurons of the AON and the HDB.

After intranasal inoculation, animals died with some regions of the brain free of virus. Mice infected with AvO1 survived, although several nuclei such as HDB or ventromedial thalamus were heavily infected. The death of CVS-infected animals is then probably due to the infection of nuclei which are permissible for CVS but not for avirulent mutants. Such observations reduce the number of potential targets for the lethal effects of the virus.

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REFERENCES

BARTHOLD, S. W. (1988). Olfactory neural pathway in mouse hepatitis virus nasoencephalitis. *Acta Neuropathol.* **76,** 502–506.

CARSON, K. A. (1984). Quantitative localization of neurons projecting to the mouse main olfactory bulb. *Brain Res. Bull.* **12,** 629–634.

COULON, P., DERBIN, C., KUCERA, P., LAFAY, F., PRÉHAUD, C., and FLAMAND, A. (1989). Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. *J. Virol.* **63,** 3550–3554.

COULON, P., ROLLIN, P., AUBERT, M., and FLAMAND, A. (1982a). Molecular basis of rabies virus virulence. I. Selection of avirulent mutants of the CVS strain with anti-G monoclonal antibodies. *J. Gen. Virol.* **61,** 97–100.

COULON, P., ROLLIN, P., BLANCOU, J., and FLAMAND, A. (1982b). Avirulent mutants of the CVS strain of rabies virus. *Comp. Immunol. Microbiol. Infect. Dis.* **5,** 117–122.

COULON, P., ROLLIN, P. E., and FLAMAND, A. (1983). Molecular basis of rabies virus virulence. II. Identification of a site on the CVS glycoprotein associated with virulence. *J. Gen. Virol.* **64,** 693–696.

DIETZSCHOLD, B., WIKTOR, T. J., TROJANOWSKI, J. Q., MACFARLAN, R. I., WUNNER, W. H., TORRES-ANJEL, M. J., and KOPROWSKI, H. (1985). Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus *in vivo* and *in vitro*. *J. Virol.* **56,** 12–18.

DIETZSCHOLD, B., WUNNER, W. H., WIKTOR, T. J., LOPES, A. D., LAFON, M., SMITH, C. L., and KOPROWSKI, H. (1983). Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. USA* **80,** 70–74.

DOLIVO, M., HONEGGER, P., KUCERA, P., KIRALY, M., and BOMMELI, W. (1981). Essai sur la structure, le métabolisme et la fonction des neurones infectés par le virus herpès suis et celui de la rage. *Rev. Inst. Pasteur Lyon* **14,** 401–419.

FEKADU, M., and SHADDOCK, B. S. (1986). Peripheral distribution of virus in dogs inoculated with two strains of rabies virus. *Am. J. Vet. Res.* **45,** 724–729.

HALASZ, N., and SHEPHERD, G. M. (1983). Neurochemistry of the vertebrate olfactory bulb. *Neuroscience* **10,** 579–619.

JACKSON, A. C. (1991). Biological basis of rabies virus neurovirulence in mice: Comparative pathogenesis study using the immunoperoxidase technique. *J. Virol.* **65,** 537–540.

KUCERA, P., DOLIVO, M., COULON, P., and FLAMAND, A. (1985). Pathways of the early propagation of virulent and avirulent rabies virus strains from the eye to the brain. *J. Virol.* **55,** 158–162.

LEHMANN, A. (1974). "Atlas Stéréotaxique du Cerveau de la Souris." Editions du Centre National de la Recherche Scientifique, Paris.

LENTZ, T. L., BURRAGE, T. G., SMITH, A. B., CRICK, J., and TIGNOR, G. H. (1982). Is the acetylcholine receptor a rabies virus receptor? *Science* **215,** 182–184.

LENTZ, T. L., WILSON, P. T., HAWROT, E., and SPEICHER, D. W. (1984).

Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. *Science* **226,** 847–848.

LUNDH, B., KRISTENSSON, K., and NORRBY, E. (1987). Selective infections of olfactory and respiratory epithelium by vesicular stomatitis virus and Sendai virus. *Neuropathol. Appl. Neurobiol.* **13,** 111–122.

LUNDH, B., LÖVE, A., KRISTENSSON, K., and NORRBY, E. (1988). Nonlethal infection of aminergic reticular core neurons: Age-dependent spread of ts mutant of vesicular stomatitis virus from the nose. *J. Neuropathol. Exp. Neurol.* **47,** 497–506.

MCLEAN, J. H., and SHIPLEY, M. T. (1987). Serotonergic afferents to the rat olfactory bulb. I. Origins of laminar specificity of serotonergic inputs in the adult rat. *J. Neurosci.* **7,** 3016–3028.

MCLEAN, J. H., SHIPLEY, M. T., and BERNSTEIN, D. I. (1989). Golgi-like, transneuronal retrograde labelling with CNS injections of herpes simplex virus type 1. *Brain Res. Bull.* **22,** 867–881.

MEISAMI, E., and SAFARI, L. (1981). Quantitative study of the effects of early unilateral olfactory deprivation on the number of mitral and tufted cells in the rat olfactory bulb. *Brain Res.* **221,** 81–107.

MURPHY, F. A., and BAUER, S. P. (1974). Early street rabies virus infection in striated muscle and later progression to the central nervous system. *Intervirology* **3,** 256–268.

MURPHY, F. A., HARRISON, A. K., and WINN, W. C., JR. (1973). Comparative pathogenesis of rabies and rabies-like viruses. Infection of the central nervous system and centrifugal spread of virus to peripheral tissues. *Lab. Invest.* **29,** 1–16.

NICKELL, W. T., and SHIPLEY, M. T. (1988). Two anatomically specific classes of candidate cholinoceptive neurons in the rat olfactory bulb. *J. Neurosci.* **8,** 4482–4491.

PAXINOS, G., and WATSON, C. (1986). "The Rat Brain in Stereotaxic Coordinates," 2nd ed., Academic Press, San Diego.

PERLMAN, S., JACOBSEN, G., and AFIFI, A. (1989). Spread of a neurotropic murine coronavirus into the CNS via the trigeminal and olfactory nerves. *Virology* **170,** 556–560.

PRÉHAUD, C., COULON, P., LAFAY, F., THIERS, C., and FLAMAND, A. (1988). Antigenic site II of the rabies glycoprotein: Structure and role in viral virulence. *J. Virol.* **62,** 1–7.

SCHNEIDER, S. P., and MACRIDES, F. (1978). Laminar distributions of interneurons in the main olfactory bulb of adult hamster. *Brain Res. Bull.* **3,** 73–82.

SEIF, I., COULON, P., ROLLIN, P. E., and FLAMAND, A. (1985). Rabies virus virulence: Effect on pathogenicity and sequence characterization of mutations affecting antigenic site III of the glycoprotein. *J. Virol.* **53,** 926–935.

SHIPLEY, M. T. (1985). Transport of molecules from nose to brain: Transneuronal anterograde and retrograde labeling in the rat olfactory system by wheat germ agglutinin–horseradish peroxidase applied to the nasal epithelium. *Brain Res. Bull.* **15,** 129–142.

SHIPLEY, M. T., HALLORAN, F. J., and DE LA TORRE, J. (1985). Surprisingly rich projection from locus coeruleus to the olfactory bulb in the rat. *Brain Res.* **329,** 294–299.

SWITZER, R. C., DE OLMOS, J., and HEIMER, L. (1985). Olfactory system. *In* "The Rat Nervous System" (G. Paxinos, Ed.), Vol. 1, pp. 1–36. Academic Press, Sydney.

TSIANG, H., DERER, M., and TAXI, J. (1983). An *in vivo* and *in vitro* study of rabies virus infection of the rat superior cervical ganglia. *Arch. Virol.* **76,** 231–243.

TUFFEREAU, C., LEBLOIS, H., BENEJEAN, J., COULON, P., LAFAY, F., and FLAMAND, A. (1989). Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. *Virology* **172,** 206–212.

WERTZ, G. W., DAVIS, N. L., and PATTON, J. (1987). The role of proteins in vesicular stomatitis virus replication. *In* "The Rhabdoviruses" (R. R. Wagner, Ed.), pp. 271–296. Plenum, New York.

WINKLER, W. C. (1975). Airborne rabies. *In* "The Natural History of Rabies" (G. M. Baer, Ed.), pp. 115–121. Academic Press, London.

ZÁBORSZKY, L., CARLSEN, J., BRASHEAR, H. R., and HEIMER, L. (1986). Cholinergic and GABAergic afferents to the olfactory bulb in the rat with special emphasis on the projection neurons in the nucleus of the horizontal limb of the diagonal band. *J. Comp. Neurol.* **243,** 488–509.