The Substantia Nigra is a Major Target for Neurovirulent Influenza A Virus

By Mitsuo Takahashi,*¶ Tatsuo Yamada,‡ Setsuko Nakajima,§ Katsuhisa Nakajima,‖ Takayuki Yamamoto,* and Hidechika Okada¶

*From *Choju Medical Institute, Noyori Fukushi-mura Hospital, Toyohashi 441, Japan; ‡Department of Neurology, School of Medicine, Chiba University, Chiba 260, Japan; §Department of Microbiology, the Institute of Public Health, Minato-ku, Tokyo 108, Japan; and the Departments of ‖Virology and ¶Molecular Biology, Nagoya City University School of Medicine, Nagoya 467, Japan*

Summary

Clinical and immunohistochemical studies were done for 3–39 d on mice after intracerebral inoculation with the neurovirulent A/WSN/33 (H1N1; WSN) strain of influenza A virus, the nonneurovirulent A/Aichi/2/68 (H3N2; Aichi) strain, and two reassortant viruses between them. The virus strains with the WSN gene segment coding for neuraminidase induced meningoencephalitis in mice. The mice inoculated with the R96 strain, which has only the neuraminidase gene from the WSN strain, had mild symptoms and weak positive immunostaining to the anti-WSN antibody in meningeal regions. Both the WSN and R404BP strains, which contain the WSN gene segments coding for neuraminidase and matrix protein, were clearly neurovirulent both clinically and pathologically. On day 3 after inoculation with either of these two strains, WSN antigen was detected in meningeal and ependymal areas, neurons of circumventricular regions, the cerebral and cerebellar cortices, the substantia nigra zona compacta, and the ventral tegmental area. On day 7, meningeal reactions and neuronal staining were still seen, and advanced accumulation of the viral antigen was evident in the substantia nigra zona compacta and hippocampus. Double immunostaining demonstrated that the WSN antigen was only seen in neurons and not in microglia or reactive astrocytes. Immunostaining for the lectin maackia amurensis agglutinin, which recognizes the Neu5Ac α2,3 Gal sequence, which serves as a binding site for influenza A virus on target cell membranes, showed that positive staining was localized in the ventral substantia nigra and hippocampus. These results suggest that neurovirulent influenza A viruses could be one of the causative agents for postencephalitic parkinsonism.

Acute, often transitory Parkinsonism has been reported during viral encephalitis. The causative viruses include Japanese encephalitis B (1, 2), coxsackie B2 (3), Western equine encephalitis (4), tick-borne encephalitis, and influenza A (5) viruses. Antigens of the influenza A virus have been detected in the brains of people with postencephalitic parkinsonism (6). Furthermore, the influenza A virus has been suspected as the causative agent of encephalitis lethargica, in which there is chronic progressive parkinsonism, because of the concurrence of that condition with the 1918–1928 influenza pandemic (7–9). An increased risk of developing idiopathic Parkinson's disease in individuals born during that influenza pandemic has also been reported (10). However, two neuropathological reports, one by Miyoshi et al. (11) and the other by Reinacher et al. (12), studying the A/NWS/33 strain of influenza A virus in mouse brains did not show any damage to the substantia nigra such as that which occurs in parkinsonism.

The human influenza A viruses expressing neurovirulence in mice are the early H1N1 strains A/NWS/33 (NWS) and A/WSN/33 (WSN). NWS and WSN strains have been maintained in many laboratories and have retained their unique pathogenic properties (13). Neurovirulence has also been reported for the reassortant strains between WSN and A/Aichi/2/68 (14, 15). Infection of mice with these viruses was induced by intracerebral inoculation that caused a meningoencephalitic condition (12).

The present studies were performed to evaluate the clin-

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ical symptoms and the localization of neurovirulent influenza A virus infection in mice after intracerebral injection. We used four different strains of influenza A virus and found clear involvement of neurons of the substantia nigra, as well as of neurons in other circumventricular nuclei.

Materials and Methods

Cell Cultures and Virus Strains. Madin-Darby canine kidney (MDCK)[1] cells were propagated and maintained as described previously (14). Virus strains used in this study and their gene derivations are presented in Table 1. The A/WSN/33 (H1N1;WSN) and A/Aichi/2/68 (H3N2;Aichi) prototype strains and preparation of recombinants between them have been described in detail previously (14). Viral infectivity was determined by plaque assay in MDCK cells (15, 16).

Mice and Viral Inoculation. Specific pathogen–free, 6 wk old male mice of the C3H/HeN inbred strain were purchased from Charles River Japan, Inc. (Tokyo, Japan). They were infected on the day after arrival and housed in cages that were placed in a safety cabinet. Each mouse received a direct intracerebral inoculation into left parietal lobe with a double-tapered needle of 0.03 ml PBS containing 5×10^3 pfu of the virus to be tested. The mice in one group received only PBS and the rest of the experimental groups received PBS containing one of the four strains used (Aichi, R96, R404BP, or WSN). The number of mice in each group was between 10 and 30 (Table 2). 3, 7, 14, and 39 d after inoculation, they were evaluated clinically, and three to five mice of each group were killed and processed for immunohistochemistry and virus plaque assays (Table 1).

Clinical Evaluations. Motor symptoms after virus inoculation, such as paresis, disequilibria, involuntary movements, or abrupt seizure-like fits, were observed daily and recorded for each mouse as described by Henle and Henle (17). The body weight of each mouse was also measured before and after inoculation. Statistical analyses were done using one-way ANOVA followed by the Scheffe's test.

Immunohistochemistry. Mice were anesthetized with sodium pentobarbital before all experiments. They were perfused through the heart with 0.01 M phosphate buffer, pH 7.4, and then with a fixative consisting of 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The brain was quickly removed and fixed for an additional 48 h at 4°C. It was then transferred to 15% sucrose in 0.1 M PBS, and serially cut at 20 μm on a freezing microtome for immunohistochemistry. Sections were rinsed three times with PBS containing 0.3% Triton X-100 (PBST) and then treated for 48 h in the cold with either anti-WSN antibody (rabbit polyclonal, 1:10,000) or anti-HK antibody (rabbit polyclonal, 1:10,000). The antibodies were prepared and well characterized by Ueda and Sugiura (18). After treatment with the primary antibody, the sections were treated for 2 h at room temperature with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) (goat anti–rabbit IgG, 1:1,000), followed by incubation in the avidin-biotinylated horseradish peroxidase complex (Vector). Washing between steps was done with PBST. Peroxidase labeling was visualized by incubating with a solution containing 0.001% 3,3'-diaminobenzidine (DAB), 0.6% nickel ammonium sulfate, 0.05% imidazole, and 0.0003% H_2O_2. When a dark purple product formed, the reaction was terminated. Sections were washed, mounted on glass slides, dehydrated with graded alcohols, and coverslipped with Entellan (Merck, Darmstadt, Germany).

Double immunostaining was done using anti-WSN antibody and one of the following antibodies: antityrosine hydroxylase (antityrosine hydroxylase [anti-TH]; Chemicon International, Inc., Temecula, CA) (mouse monoclonal, 1:1,000), antimacrophage (Boehringer Mannheim GmbH, Mannheim, Germany) (Mac-1, rat monoclonal, 1:200), and antiglial fibrillary acidic protein (DAKO, Glostrup, Denmark) (GFAP, rabbit polyclonal, 1:10,000). Sections were treated for 30 min with 0.5% H_2O_2 solution in PBST after the DAB reaction of the first cycle. The second cycle was carried out in a manner similar to the first, except that nickel ammonium sulfate was omitted from the DAB solution, yielding a brown precipitate in the second cycle. Further details on the immunohistochemical procedures have been published previously (19).

Digoxigenin-labeled lectins of sambucus nigra agglutinin (SNA) and maackia amurensis agglutinin (MAA) were used to detect the carbohydrate moieties of glycoproteins serving as influenza A virus receptors. The MAA recognizes the sequence Neu5Ac α2,3Gal and the SNA is specific for the Neu5Ac α2,6Gal sequence. Slices were incubated in blocking solution (1% skim milk) at 4°C overnight to avoid nonspecific binding. The subsequent procedures were strictly according to the method recommended in the DIG Glycan Differential Kit from Boehringer Mannheim (20).

Virus Plaque Assay. Freshly frozen brain was ground in a motor-driven glass homogenizer and a suspension was prepared in 2 ml of PBS. The suspension was used to determine the content of infectious virus by plaque assay in MDCK cells (15, 16).

Results

Clinical Evaluations

Virus inoculation was achieved in all mice by intracerebral route without acute complications of seizure or respiratory arrest. Brain samples obtained for immunohistochemistry (fixed) and for plaque assay (frozen) in this study are listed in Table 1. Table 2 shows the body weight changes during the observation period.

3 d after inoculation, the mice inoculated with PBS or Aichi/68 virus showed little difference in their behavior and were eager to eat. The R96 group showed a slight but insignificant decrease in motor activity and body weight compared to PBS mice. All mice in the R404BP and WSN groups lost body weight (Table 2) and showed decreased motor activity. All showed ruffled fur and frequent shivering. They ate poorly and were severely emaciated. Hyperirritability was also seen when stimuli of noise, flashlight, or touch were given. When suspended by their tails, almost all of the R404BP group and some of the WSN group showed marked tremulous movements and clonic convulsions that sometimes changed suddenly into tonic convulsions. When they were not being stimulated, the mice huddled in groups and kept still until the application of other noxious stimuli. Mice that showed convulsive movements developing into generalized clonic-tonic convulsions were prone to die within the next few days.

[1] *Abbreviations used in this paper:* anti-TH, antityrosine hydroxylase; CA, cornu ammonius; DAB, 3,3'-diaminobenzidine; GFAP, glial fibrillary acidic protein; HPC, hippocampus; M, matrix protein; MAA, maackia amurensis agglutinin; MDCK, Madin-Darby canine kidney (cells); NA, neuraminidase; PBST, PBS containing 0.3% Triton X-100; SN, substantia nigra; SNA, sambucus nigra agglutinin; SNC, substantia nigra zona compacta; SNR, substantia nigra zona reticulata; VTA, ventral tegmental area.

Table 1. *Prototype and Recombinant Virus Strains Used in This Study and the Number of Brain Samples in Each Experiment*

Virus strains	Genotype								Number of samples (fixed/frozen)			
	P_1	P_2	P_3	HA	NP	NA	M	NS	3 d	7 d	14 d	28 d
Aichi	H	H	H	H	H	H	H	H	3 / 2	3 / 2	3 / 2	3 / 2
R96	H	H	H	H	H	W	H	H	3 / 2	3 / 2	3 / 2	3 / 2
R404BP	H	H	H	H	H	W	W	H	3 / 2	2 / 0*	NO	NO
WSN	W	W	W	W	W	W	W	W	3 / 2	3 / 2	3 / 2	2 / 1

Genes derived from WSN and Hong Kong (HK) are indicated by W and H, respectively (14).
P_1, P_2, P_3, polymerase; HA, hemagglutinin; NP, nuclear protein; NA, neuraminidase; M, matrix protein, NS, nonstructural protein; NO, not obtained.
* These samples were obtained on day 6.

Within 7 d after inoculation, all 20 mice in the R404BP group and 10 out of 30 mice of the WSN group died with acute meningo-encephalitis. On day 6, we killed the three surviving mice in the R404BP group because they showed general prostration. The WSN group showed marked body weight loss on days 7 and 14 (Table 2) and the most severe clinical symptoms seen during the observation period on day 7. The mice in the WSN group surviving beyond 7 d after inoculation showed gradual improvement in motor activity and body weight.

During the early time period (3–14 d), there was a clear clinical distinction between mice in the R404BP and WSN groups, which showed meningo-encephalitis, and those in the PBS, Aichi/68, and R96 groups, which were either neurologically normal or very mildly affected. Within 3 or 6 wk after inoculation, however, the difference in the clinical symptoms between the groups could not be observed in surviving mice. On day 39, the WSN group was neurologically normal.

Immunohistochemical Study with Anti-WSN Antibody

Of the brains taken for immunohistochemical study, only those from the WSN and R404BP groups showed clear evidence of viral invasion. The brains from the R96 group showed only limited staining to anti-WSN antibody in the meninges, ependymal cells, and neurites. No pathological changes were seen in the PBS and Aichi/68 groups, except in the parietal cortex of the needle wounds. The neuropathological findings in the R404BP and WSN groups were predominantly in the meningeal, circumventricular, cortical, and nigral regions. Other parts of their brains showed only minor staining with marked differences from mouse to mouse.

3 d after Inoculation in the WSN and R404BP Groups. Diffuse and granular staining were clearly seen in arachnoid, pia mater, and ependymal cells in all mice. Reflecting the direct intracerebral route of the virus' entry, prominent involvement was mainly seen in the circumventricular regions, such as the hippocampus (HPC), hypothalamus, periaqueductal grey matter, and the regions surrounding the fourth ventricle. Focal invasion of the WSN strain could be seen in hippocampal tissues (Fig. 1 *A*). Some neurons in the granular cell layer of the dentate gyrus with fine neurites connecting to the alveus were positively stained, as were a few neurons in the amygdala. Neurons in the anterior, lateral, and ventromedian hypothalamic nucleus were sometimes stained positively, and a few neurons in the locus ceruleus

Table 2. *Mean (±SD) Body Weights in the Groups with PBS and Four Different Viruses*

Groups	Body weight (g)				
	Before	3 d	7 d	14 d	39 d
PBS (*n*)	22.6 ± 0.6 (10)	24.7 ± 0.4 (10)	25.6 ± 0.5 (7)	26.8 ± 0.4 (4)	30.6 ± 1.5 (3)
Aichi (*n*)	23.3 ± 1.3 (20)	25.8 ± 1.1 (20)	26.7 ± 0.6 (15)	27.0 ± 0.9 (10)	30.2 ± 1.1 (5)
R96 (*n*)	22.7 ± 1.2 (20)	24.9 ± 1.1 (20)	26.3 ± 1.3 (15)	25.2 ± 0.8 (10)	30.1 ± 1.9 (5)
R404BP (*n*)	23.0 ± 1.4 (20)	21.8 ± 1.4 (20)*			
WSN (*n*)	23.0 ± 1.3 (30)	21.5 ± 1.6 (30)*	16.2 ± 1.0 (15)*	16.5 ± 1.4 (8)*	27.1 ± 1.8 (5)

n, number of mice.
* Significantly different ($P < 0.01$) from the PBS group.

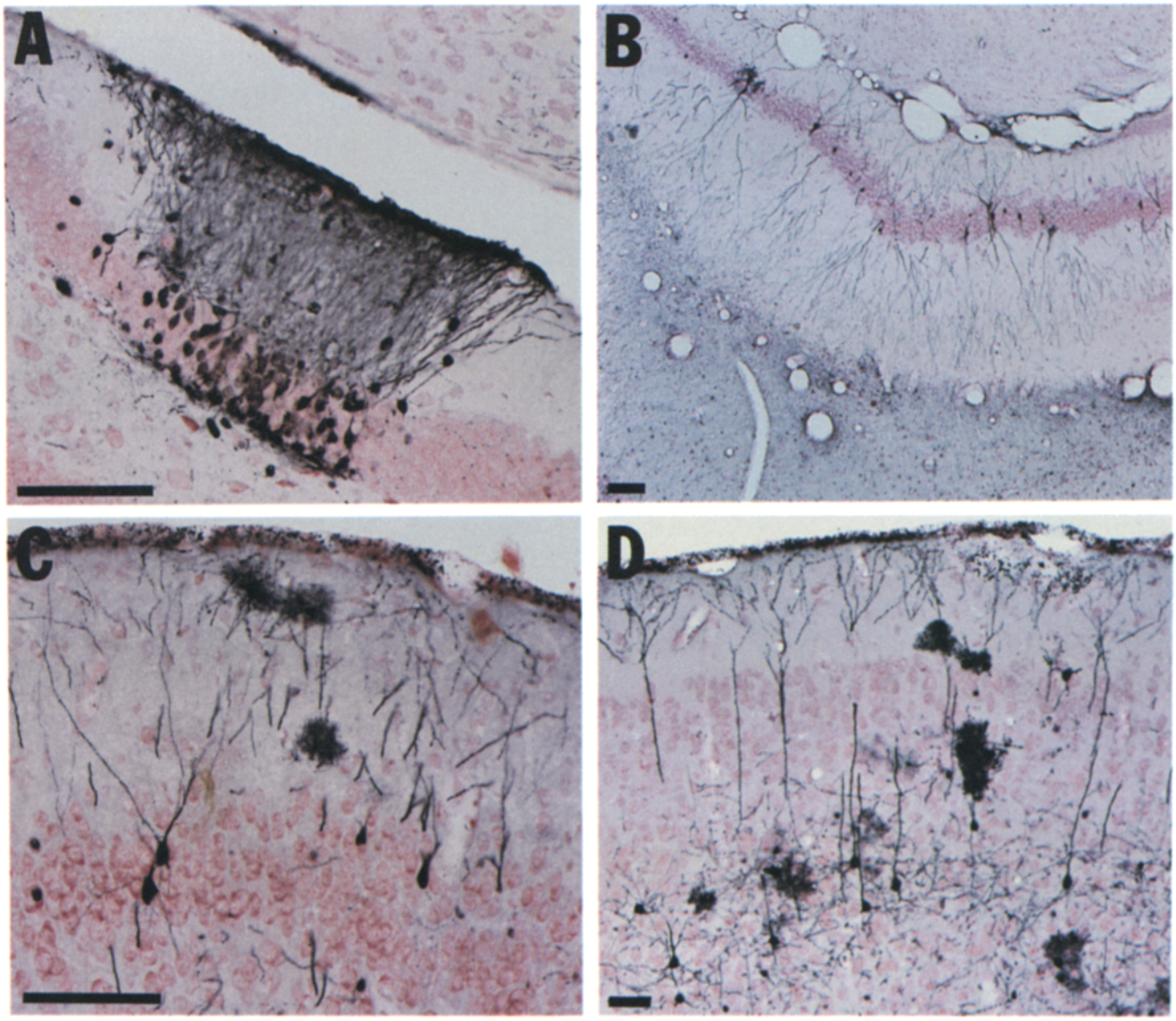

Figure 1. Immunohistochemistry with the anti-WSN antibody, counterstained with neutral red, in the HPC of mice from the R404BP group on day 3 (*A*) and day 7 (*B*), and in the parietal cortex of mice in the WSN group on day 3 (*C*), and day 7 (*D*). (*A*) Positively stained neurons in the granular cell layer of the dentate gyrus with many neurites reaching the surface ependymal cell layer are seen. However, they show only focal immunostaining. (*B*) Many neurons in the pyramidal cell layer are stained positively. (*C*) Diffuse and granular staining is seen in the pia mater. In the parenchyma, a few neurons and many neurites are positive to the anti-WSN antibody. A few irregularly shaped, diffusely labeled areas containing positively stained neurons are also seen in the cortex. (*D*) The immunostaining is almost the same as on day 3 (see *C*). Bar, 100 μm.

located on the lateral wall of the fourth ventricle were sometimes mildly stained. This neuronal staining varied from mouse to mouse, showing no constant pattern.

In the cerebral neocortex, positively stained neurons and neurites were scattered in the parieto-occipital regions, giving evidence of the route of viral invasion (Fig. 1 *C*). A few irregularly immunopositive areas that contained positively stained neurons and surrounding neuropils were also seen (Fig. 1 *C*). In the cerebellar cortex, a small number of Purkinje cells and their dendrites were strongly stained. The other parts of cerebellum showed no immunostaining.

Constant and reproducible positive staining was seen in the neurons of the substantia nigra (SN) and ventral tegmental area (VTA) (Fig. 2, *A* and *B*). Neurites in the substantia nigra zona reticulata (SNR) were also stained (Fig. 2 *B*).

6 and 7 d after Inoculation. During this period, meningeal involvement was still seen. The major pathology was, however, in the deep cortex and white matter (Fig. 3).

All of the cornu ammonius 1 (CA 1) and CA 2 showed diffuse immunolabeling of neuronal cells with numerous positive branching neurites (Fig. 1 *B*). In CA 3, only a small number of neurons were stained. The dentate gyral lesions had subsided and staining of the medial hypothalamic area was decreased compared to that seen on day 3. Neurons in the central gray surrounding the aqueduct and the other circumventricular regions showed the same staining pattern as

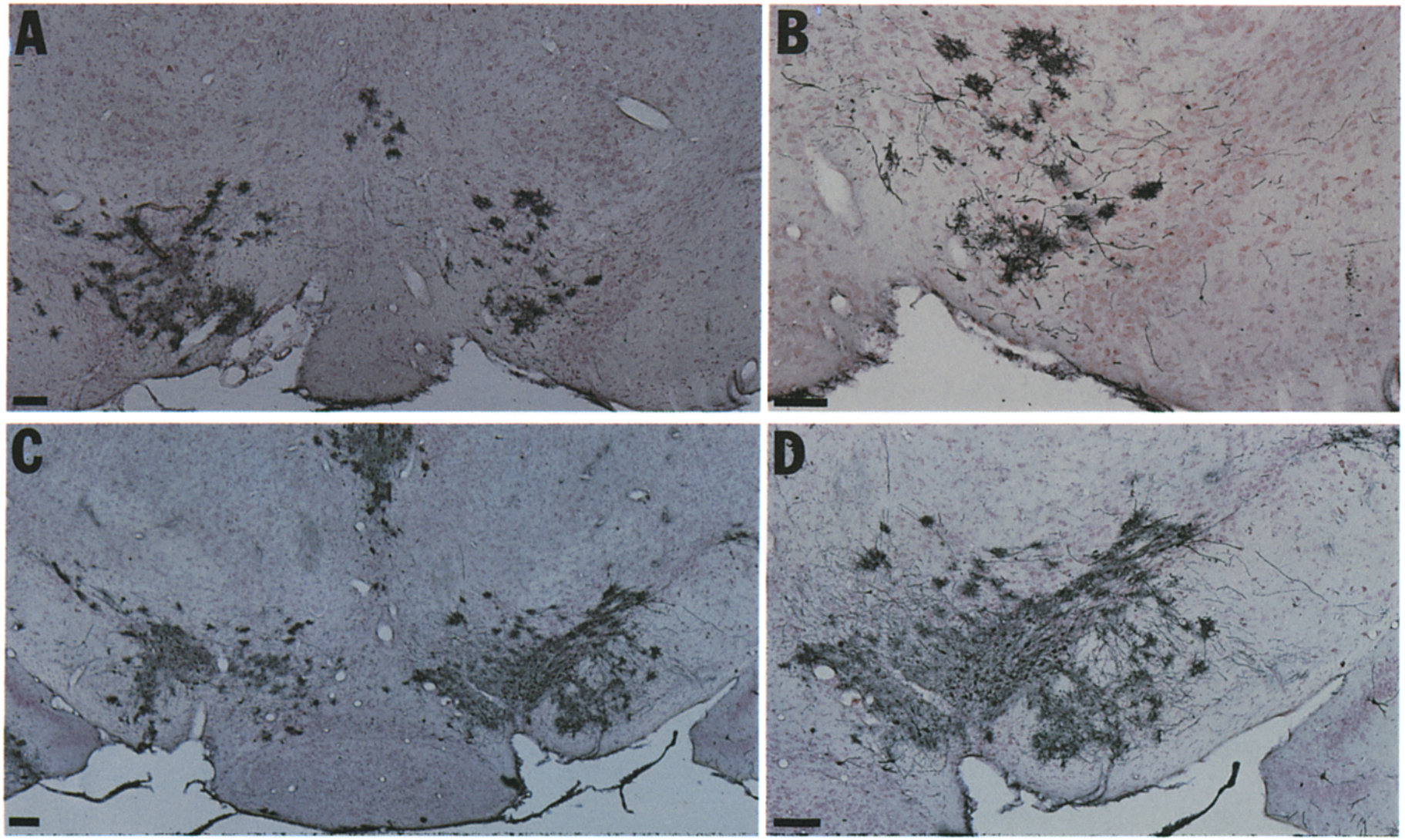

Figure 2. Immunohistochemistry using the anti-WSN antibody in mice from the R404BP group on day 3 (*A* and *B*) and day 7 (*C* and *D*). (*A*) Low power photomicrograph demonstrating WSN antigen in the bilateral medial parts of the SN and VTA. (*B*) High power photomicrograph of *A* shows that the positive immunostaining is localized in some neurons, neurites, and the neuropil surrounding them. (*C*) Low magnification photomicrograph indicating more prominent staining in the SN than on day 3, now extending to the SNC laterally and SNR ventrolaterally. Additional labeling is seen in the central gray. (*D*) Higher magnification of *C* shows that a large number of neurons with neurites extending dorsolaterally in the SNC are strongly positive. Bar, 100 μm.

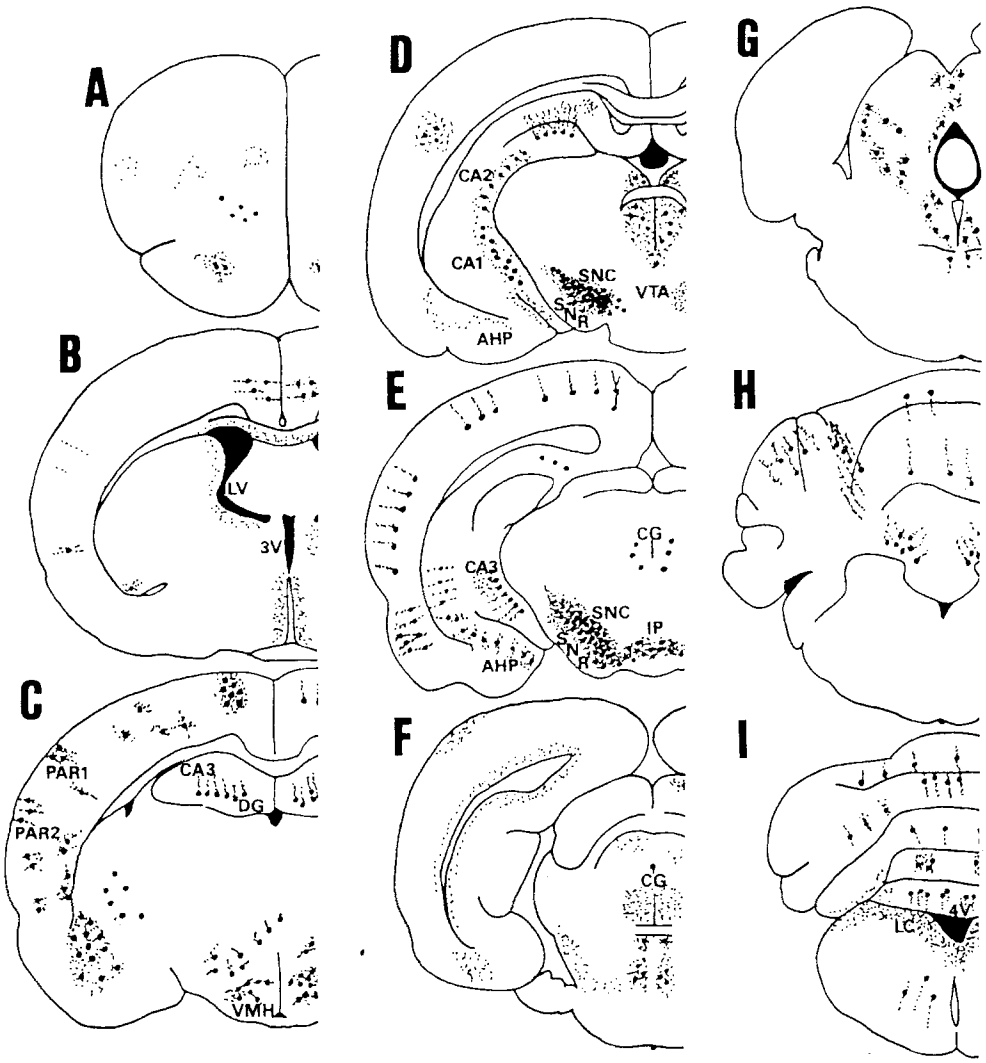

Figure 3. A series of line drawings of sections through the brain to illustrate the distribution of immunostaining to anti-WSN antibody seen in a mouse of the R404BP group on day 6 after inoculaton. The large dots represent labeled neuronal perikarya. The small dots indicate the labeling of neurites. Structures are named according to the atlas by Paxinos and Watson (21).

on day 3. A few neurons in the locus ceruleus were also stained.

In the neocortex, some areas showed mild neuronal staining throughout all the cortical layers (Fig. 2 *D*). In the cerebellum, less immunostaining was seen in Purkinje cells and their dendrites than on day 3.

The neuronal involvement in the SN, especially the substantia nigra zona compacta (SNC), was more advanced. Marked staining were seen in the neurons of the SN and VTA. Many neurons in the VTA and almost all of the neurons in the SNC showed strong positive immunostaining (Fig. 2, *C* and *D*). The axons extending to the striatum were also stained. Neurites in the SNR, which may be dendrites from the SNC neurons, and some neurons in the interpeduncular nucleus were also clearly stained. Many cotton-like, irregularly round-shaped areas of staining were seen in the SN, probably reflecting a seepage of the WSN antigen through the neuropil (Fig. 2 *D*). Every sample obtained from the WSN and R404BP groups had the same immunohistchemical pattern in the nigral region, and this was the most striking pathological change in the whole brain.

2 wk and beyond after Inoculation. No new pathological changes or lesions were detected (data not shown) during these time periods. No viral antigen was observed in any brain areas of mice that survived up to 4 wk after inoculation.

Double Immunostaining

Double immunostaining with anti-WSN and anti-TH antibodies showed that a large number of TH-positive nigral cells had the WSN antigen. Some TH-positive neurons contained a WSN-positive nucleus (Fig. 4 *C*), suggesting early nuclear invasion of the virus. Double staining with anti-WSN and anti-Mac 1 antibodies (Fig. 4 *D*), or with anti-WSN and anti-GFAP antibodies (Fig. 4 *E*), revealed that the positive cells were neither astrocytes nor microglia. Morphologically, all the positive cells appeared to be neurons.

Lectin Immunostaining

Staining with digoxigenin-MAA gave considerable labeling over the ventral surface of the SN (Fig. 4 *A*) and the hippocampal formation. In the cerebellum, the molecular layer and Purkinje cells were strongly stained (Fig. 4 *B*). The other

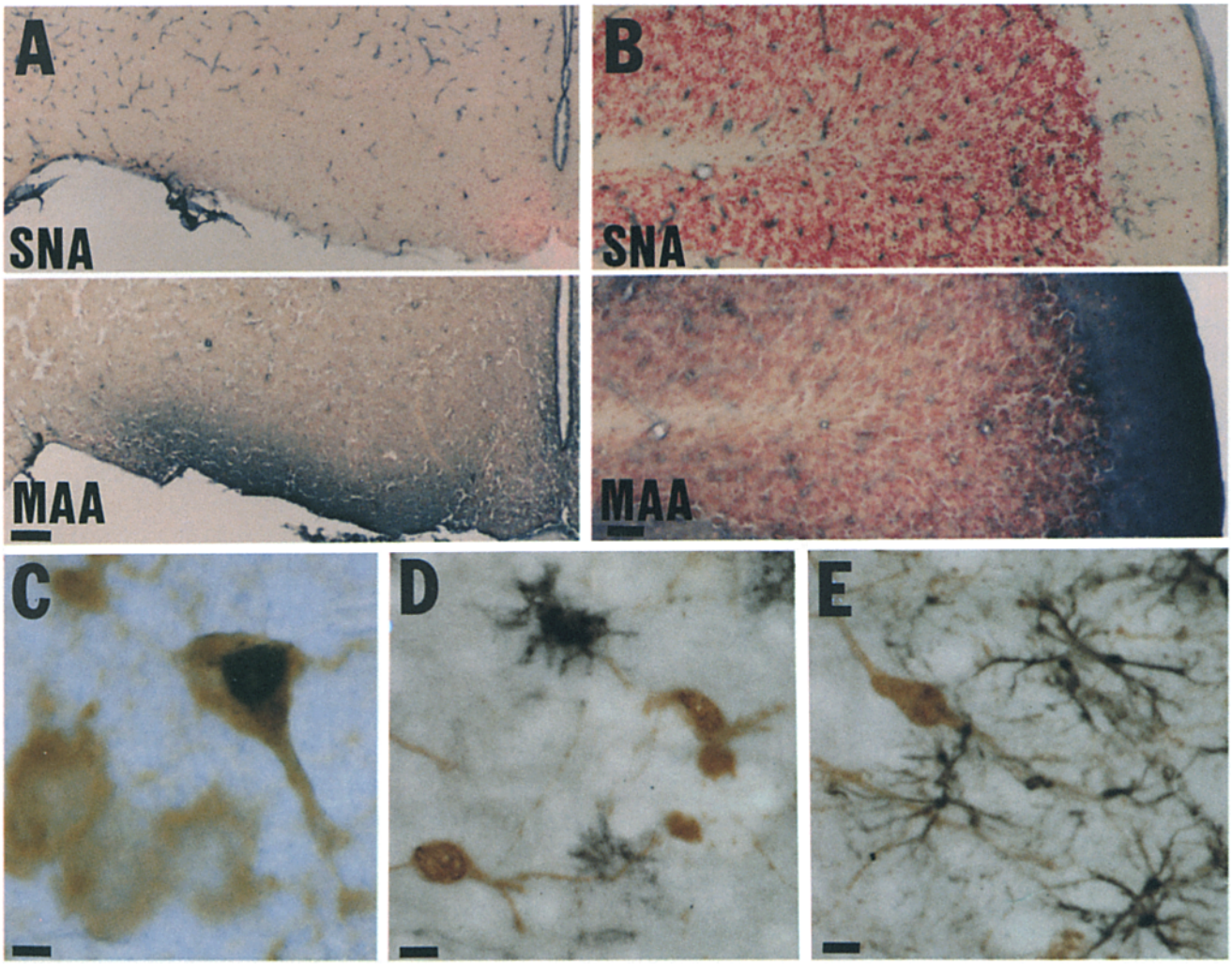

Figure 4. Immunohistochemistry with the antibody to lectin in mice treated with PBS. (*A*) In a section through the optic chiasma, the ventral portion adjacent to the SN is diffuse but densely stained for the lectin MAA, whereas the corresponding region for the lectin SNA is not stained at all. (*B*) Staining for MAA shows diffuse immunolabeling in the molecular cell layer and Purkinje cells of the cerebellum. In contrast, staining for SNA shows no specific immunolabeling. Double immunostaining in mice from the WSN group on day 7 (*C–E*) Double immunostaining with anti-WSN and anti-TH antibodies (*C*) showed that a TH-positive neuron (*brown*) in the SN contains a strongly WSN-positive nucleus and faintly positive cytoplasm (*dark blue*). Double immunostaining with anti-WSN and anti–Mac-1 antibodies (*D*), or with anti-WSN and anti-GFAP antibodies (*E*), showed that WSN-positive structures (*brown*) were not stained with either the anti–GFAP or anti–Mac-1 antibody (*dark blue*). Bar in *A* and *B*, 100 μm, bar in *C–E*, 10 μm.

areas of the cerebellum were not stained. In contrast, staining with digoxigenin-SNA did not give any positive reaction (Fig. 4, *A* and *B*).

Virus Plaque Assay

Virus replication was assessed by measuring the brain virus titer on day 14 in groups with surviving mice. Because of the inhibitory effect of brain suspensions, the minimal virus titer detectable was 20 pfu per brain. Virus content in the brains of mice infected with Aichi or R96 viruses could not be detected by this method. In contrast, the brains infected with WSN virus had a titer of 1.2×10^4 pfu/ml. This indicated that the WSN strain showed an adaptation to the mouse brain at a period when the neurological signs had largely subsided.

Discussion

We used four different recombinants between the neurovirulent WSN and the nonneurovirulent Aichi/68 strains of influenza A virus. Three genes of the WSN strain, NA, and nonstructural protein, appear to be responsible, to different degrees, for the neurovirulence in mice (14, 15). Since the R404BP strain was equivalent in neurovirulence to the WSN strain, NS seems to be less significant for neurovirulence than NA and M (22). The mild clinical symptoms and rapid recovery of the R96 strain suggest that M of the WSN virus may be responsible for efficient virus multiplication in the mouse brain. Our clinical and pathological findings indicate that NA of the WSN virus plays a dominant role for viral infection. Furthermore, the results of the virus plaque assays suggest that only WSN could adapt and replicate in the mouse

brain. All of these results are consistent with previous reports (14, 15).

The major finding in this study is that the targets of neurovirulent influenza A virus are the SN, VTA in the brainstem and the HPC. The WSN antigens were distributed not only in the neurons, but also in surrounding areas, suggesting viral seepage from the neurons. The definite and progressive involvement of the SN and HPC, especially of the SNC, showed a selective and reproducible invasion of influenza A virus into parenchymal tissues of the mouse brain. Furthermore, there was early nuclear invasion by neurovirulent influenza A virus in some dopaminergic neurons in the SNC. Although our experiments are limited to the acute phase, this result suggests that the SNC is vulnerable to attack by neurovirulent influenza A virus.

The hemagglutinin glycoproteins of influenza viruses bind to cell membrane receptor molecules that contain sialic acid, such as glycoproteins (23) and gangliosides (24). Every strain of influenza virus has its particular preference in sialic acid–binding properties. Human influenza virus A/PR/8/34 (H1N1), which has the same serotype as the WSN strain, recognizes Neu5Ac α2, 3Gal more specifically than it does Neu5Ac α2, 6Gal, whereas A/Aichi/68 has the reverse pattern (25). Lectin immunostaining showing positive reactions in the SN, HPC, and cerebellar cortex, where intense immunolabelings to WSN antigen were also observed, suggesting that the neurovirulence of WSN strain and its localization in mice may depend partly on the distribution of Neu5Ac α2, 3Gal as a virus receptor (26). There are probably other factors determining whether influenza virus can attach to host cell membrane and fuse with it to penetrate the cytoplasm after incorporation by receptor-mediated endocytosis (27). One such factor may be cleavage of hemagglutinin by a certain kind of trypsin-like protease to transform it into the active subunit for fusion (28). Further study is necessary to clarify the significance of these factors in the neuronal involvement.

Analysis of the nucleotide sequences of eight influenza A virus RNA segments indicates that all of the influenza viruses in mammalian hosts originate from the gene pool in the aquatic avian reservoir. There are periodic exchanges of influenza virus genes or whole viruses between species, giving rise to pandemics in humans (29, 30). In the 20th century, there have been four such pandemics (1918–1919, 1957–1958, 1968–1969, and 1977), caused by the emergence of new subtypes of the influenza A virus. The great pandemic of 1918–1919, caused by the serotype of H1N1, caused an estimated 20 million deaths (31). Encephalitis lethargica was observed worldwide between 1915 and 1928 and then declined, with only very occasional reports of encephalitis cases after 1930. However, its sequela, postencephalitic parkinsonism/amyotrophic lateral sclerosis (especially parkinsonism), was observed during the next two to three decades. Because of the concurrence of the 1918 influenza pandemic and a pandemic of encephalitis lethargica beginning with flu-like episodes, the influenza virus has been thought by a number of investigators to be the cause of encephalitis lethargica. Virus localization in this study, using the same H1N1 serotype, suggests that the influenza A virus has a strong potency to cause postencephalitic parkinsonism in humans.

Several authors have hypothesized that influenza A virus infection may be a cause of Parkinson's disease (32–37). The fact that the WSN strain of influenza A virus has an affinity to the SN, as reported here, supports this hypothesis. Via the natural route of infection, the blood–brain barrier should prevent virus invasion of the brain via blood vessels from the infected lung. Furthermore, it would be difficult for influenza A virus–infected cells to survive complement-mediated cell damage in blood circulation since virus-infected cells are reactive to homologous complement. Therefore, a possible route of infection to the brain may be via the olfactory nerve system and this possibility is one of the most important questions to be answered. In the present experiment, we could not obtain chronic infection in the mouse brain. There remains the possibility of establishing new models showing chronic and persistent influenza A virus infection in the brain, which could help to clarify the relationship between postencephalitic parkinsonism and Parkinson's disease.

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Address correspondence to Dr. Hidechika Okada, Nagoya City University School of Medicine, Department of Molecular Biology, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan.

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