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Genetic contributions to influenza virus attenuation in the rat brain

Li Qi,[1] Kathryn M Carbone,[1] Zhiping Ye,[1] Teresa Liu,[1] Mikhail Ovanesov,[2] Mikhail Pletnikov,[2] Christian Sauder,[1] and Steven A Rubin[1]

[1]CBER, Food and Drug Administration, Bethesda, Maryland, USA; [2]Department of Psychiatry, Johns Hopkins University, Baltimore, Maryland, USA

Influenza is generally regarded as an infection of the respiratory tract; however, neurological involvement is a well-recognized, although uncommon, complication of influenza A virus infection. The authors previously described the development of a rat model for studying influenza virus infection of the central nervous system (CNS). This model was used here to study the role of virus genes in virus replication and spread in brain. In the present work, an infectious cDNA clone of the neurotoxic WSN strain of influenza virus (rWSN) was altered by site-directed mutagenesis at five loci that corresponded to changes previously shown to confer temperature sensitivity and attenuation of the A/Ann Arbor/6/60 strain (PB1Δ391, PB1Δ581, and PB1Δ661; PB2Δ265, and NPΔ34). Whereas rWSN and its mutated derivative (mu-rWSN) replicated equally well in MDCK cells at 37°C (the body temperature of rats), rWSN grew to higher titers and infection was more widespread compared to mu-rWSN in rat brain. These results demonstrate that the five mutations that confer attenuation of the A/Ann Arbor/6/60 influenza virus strain for the respiratory system also confer attenuation for the central nervous system. Further *in vivo* and *in vitro* examination of these five mutations, both individually and in combination, will likely provide important information on the role of specific virus genes in virulence and pathogenesis. *Journal of NeuroVirology* (2008) **14,** 136–142.

Keywords: attenuation; influenza virus; neurotoxicity; rat

Introduction

Influenza is generally regarded as an infection of the respiratory tract; however, influenza-associated encephalopathy and other neurological abnormalities have been well documented (Kristensson, 2006). Although rare, direct virus invasion of the central nervous system (CNS) does occur, as evidenced by detection of virus in the brain and the cerebrospinal fluid of patients with clinically diagnosed influenza-associated encephalopathy (Frankova *et al*, 1977; Murphy and Hawkes, 1970; Rose and Prabhakar, 1982; Thraenhart *et al*, 1975; Mori *et al*, 1999; Fujimoto *et al*, 1998; Takahashi *et al*, 2000; McCullers *et al*, 1999). In experimentally infected mice, virus has been shown to reach the brain through the olfactory and trigeminal nerve system, likely via free nerve endings near influenza virus–infected epithelial cells in the upper respiratory tract (Reinacher *et al*, 1983; Mori and Kimura, 2001). That live, attenuated influenza virus vaccines are administered intranasally, a route potentially providing access to the brain via the olfactory nerves, suggests the need for preclinical neurological safety testing of live, attenuated influenza virus vaccines. Indeed, the European Union's Committee for Medicinal Products guideline on live, attenuated influenza vaccine requires that absence of

Address correspondence to Steven A Rubin, DVP/OVRR/CBER/FDA, Building 29A, Room 1A-21, 8800 Rockville Pike, Bethesda, MD 20892, USA. E-mail: rubins@cber.fda.gov

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neurovirulence be demonstrated for live, attenuated influenza virus vaccines (The European agency for the evaluation of medicinal products, 2003; International Association for Biologicals, 2006). To respond to this need, we previously developed a prototype rat-based assay in which influenza virus neurotoxicity for humans can be examined (Rubin *et al*, 2004). Although influenza virus does not lead to overt clinical disease in rats, the relative human neurotoxicity potential of influenza virus strains could be distinguished in rats based on infectivity and spread in the brain. Further, the affected brain regions, cell types, and neuropathological changes observed in experimentally inoculated rats paralleled many of the reported features of influenza virus infection of the human central nervous system (CNS) (Rubin *et al*, 2004).

In this report, we use the rat model to explore genetic contributions to influenza virus neurotoxicity. To this end we obtained a molecular clone of the highly neurotoxic A/WSN/33 (rWSN) influenza virus strain (Hoffmann *et al*, 2000) and introduced by site-directed mutagenesis genomic changes corresponding to specific amino acid residues associated with attenuation of the A/Ann Arbor/6/60 influenza A virus strain for the respiratory tract in ferrets. The rescued virus had a K391E, E581G, and A661T change in the PB1 gene, a N265S change in the PB2 gene, and a D34G change in the NP gene (Jin *et al*, 2003; Liu and Ye, 2005). Notably, the A/Ann Arbor/6/60 influenza A virus strain is used as the master donor virus in the live, attenuated FluMist vaccine (Maassab, 1967; Jin *et al*, 2003).

Whereas the mutated WSN infectious clone (mu-rWSN) expressing the aforementioned five mutations replicated transiently and was restricted to the choroid plexus and ventricular ependymal cells of the brain in rats inoculated intracerebrally as newborns, the wild-type parental WSN infectious clone (rWSN) was able to spread to neurons deeper into the brain parenchyma and persisted longer. The differential ability of the two viruses to replicate in neurons was confirmed in primary cortical neuron/glial cultures prepared from embryonic rat brain. Whereas these cultures were permissive to infection by rWSN, no evidence of infection by mu-rWSN was noted. Importantly, the observed neuroattenuation phenotype of the virus was not due to an inability of the virus to replicate at 37°C (the temperature of rat brain), because at this temperature, both rWSN and mu-rWSN replicated to equivalent peak titers with similar kinetics in MDCK cells *in vitro*. Our results show that the same genomic mutations that result in attenuation of A/Ann Arbor/6/60 for the respiratory tract also confer neuroattenuation to WSN. Further, these are the first data demonstrating the role of the influenza virus internal genes (PB1, PB2, and NP) on neuroattenuation. Such information can be used to benefit our understanding of influenza virus pathogenesis and may lead to improved quality control and assurance of vaccine safety.

Results

The temperature sensitive phenotype of the rescued virus mu-rWSN (containing a K391E, E581G, and A661T change in the PB1 gene, a N265S change in the PB2 gene, and a D34G change in the NP gene) was confirmed by a greater than 2 log reduction in peak virus titer at 39°C versus 37°C in MDCK cells (data not shown). No significant reduction in the titer of wild-type virus rWSN was observed at 39°C. Despite the temperature sensitivity of mu-rWSN, both rWSN and mu-rWSN replicated to equivalent titers with equivalent kinetics at 37°C in MDCK cells, the temperature of rat brain, as shown in Figure 1. By day 5, the entire monolayer was lysed in both virus infected cultures. To distinguish *de novo* virus growth from persistence of virus, the stability of each virus was calculated. Aliquots of rWSN and mu-rWSN containing 5.0×10^4 plaque-forming units (PFU)/ml (equivalent to the dose of virus used to infect the MDCK cells) were incubated at 37°C in the absence of cells. Infectious virus could not be recovered from either virus preparation after 24 h under these conditions (data not shown). This indicates that the virus titers measured in MDCK cells 24 h post infection and beyond likely represent *de novo* virus production as opposed to persistence of virus from earlier time points or recovery of the original inoculum.

In contrast to the *in vitro* data where both viruses replicated similarly, in rat brain, mu-rWSN was undetectable after day 2, whereas rWSN continued to be recovered through day 4 post inoculation (Figure 2). The rWSN virus titers measured on days 2, 3, and 4 post inoculation are unlikely to simply reflect

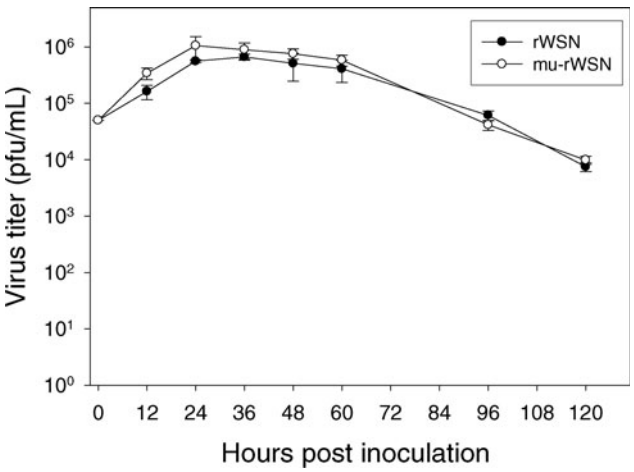

Figure 1 Cumulative growth kinetics of rWSN and mu-rWSN in MDCK cells, infected at an m.o.i. of 0.01 and incubated at 37°C. Errors bars show the standard error of the mean of three independent determinations.

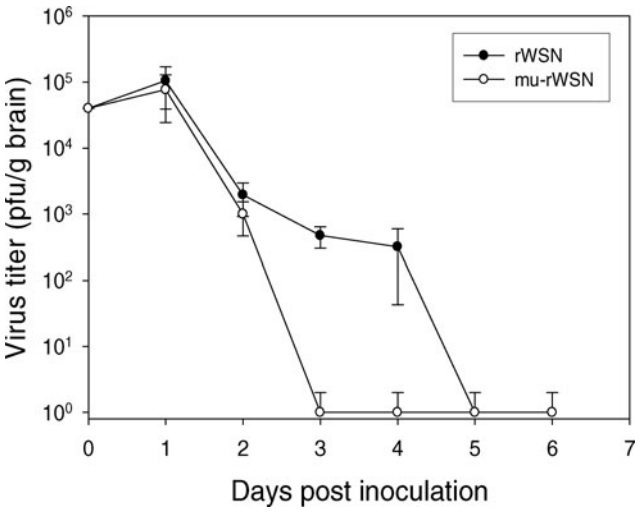

Figure 2 Virus concentration in rat brain following intracranial inoculation with 10^4 PFU of rWSN and mu-rWSN. Errors bars show the standard error of the mean of three to five independent determinations.

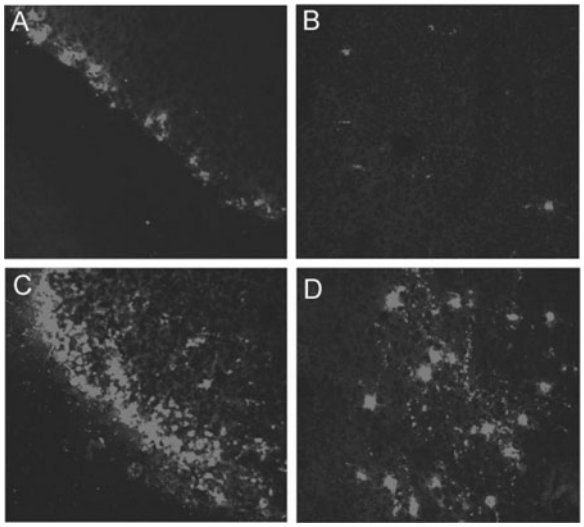

Figure 3 Sagittal sections of rat brain tissue immunohistochemically stained for influenza virus nucleoprotein three days post intracranial inoculation with mu-rWSN (**A** and **B**) or rWSN (**C** and **D**). mu-rWSN was mostly restricted to the ependymal cell lining of the ventricular system (**A**), with little to no evidence of infected areas deeper in the brain parenchyma (**B**). In contrast, rWSN heavily infected periventricular areas of the brain (**C**) and adjacent sites, including the dorsal lateral subventricular zone (**D**).

persistence of input virus, because, as suggested by the *in vitro* study described above, in the absence of a cell substrate, virus potency rapidly decays to undetectable levels with 24 h of incubation at 37°C (the temperature of rat brain).

No obvious signs of disease were noted in any of the neonatal inoculated rats throughout the course of the experiment. Hematoxylin and eosin–stained brain tissue sections did not reveal any evidence of gross neuropathology.

Immunohistochemical staining of rat brain tissue sections using an influenza virus nucleoprotein–specific monoclonal antibody showed that mu-rWSN was mostly restricted to the ependymal cell lining of the ventricular system, whereas rWSN spread to areas deeper in the brain parenchyma, including the dorsal lateral subventricular zone (Figure 3), consistent with our previous findings (Rubin *et al*, 2004). Double labeling with antibodies to astrocytes (glial fibrillary acid protein [GFAP]) or neurons (microtubule-associated protein [MAP]-2) revealed neurons were the predominant extraventricular cell type infected by influenza virus, confirming our previous report (data not shown) (Rubin *et al*, 2004).

The reduced capacity of mu-rWSN to infect and spread in the brain, relative to that observed with rWSN, was confirmed *in vitro* using mixed neuron-glia cultures prepared from rat embryos. These cultures consist of approximately 50% atrocytes, 40% neurons, and 10% microglia and other cell types, as demonstrated previously (Kim *et al*, 2000; Liu *et al*, 2000). As shown in Figure 4, these primary cultures were more permissive to rWSN than to mu-rWSN. The overwhelming majority of the infected cells in both rWSN-and mu-rWSN–inoculated cultures were identified as neurons (Figure 5).

Discussion

The identification of genetic contributions to influenza virus virulence has, for the most part, been based on studies of reassortants derived from crosses between virulent and avirulent virus strains. However, that virulent reassortants can be generated from avirulent parent strains, and vice versa, demonstrates that virulence is a polygenic trait, which confounds such studies (Dybing *et al*, 2000; Scholtissek *et al*, 1979; Brown, 2000). To properly assess the influence

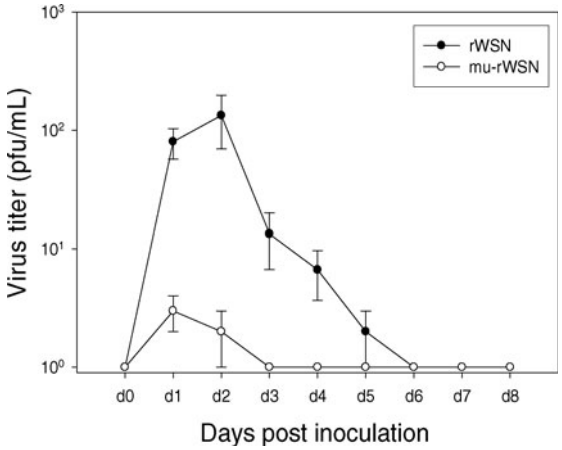

Figure 4 Cumulative growth kinetics of rWSN and mu-rWSN in mixed neuron-glial cultures prepared from cortices of embryonic day 15 to 17 Lewis rats, infected at an m.o.i. of 1.0 and incubated at 37°C. Errors bars show the standard error of the mean of three independent determinations.

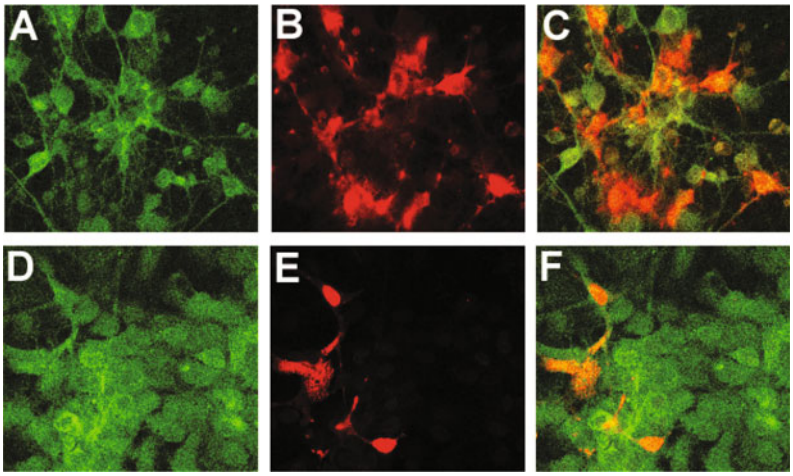

Figure 5 Confocal laser scanning microscopy of mixed primary rat neuron-glial culture 2 days post inoculation with rWSN (*top panels*, **A–C**) and mu-rWSN (*bottom panels*, **D–F**) at an m.o.i. of 1.0, showing colocalization of virus antigen with neurons. The representative cultures were dual stained with antibody directed to microtubule-associated protein-2 (**A** and **D**, *green*) and influenza virus nucleoprotein (**B** and **E**, *red*). The coincident confocal images (**C** and **F**) demonstrate colocalization of the two antigens.

of specific genomic changes on virulence, studies must be carried out with viruses of identical genetic backgrounds, i.e., use of plasmid-based reverse genetics techniques. Jin *et al* have used this approach to map the mutations responsible for the attenuation phenotype of the temperature sensitive A/Ann Arbor/6/60 strain, the master donor virus for the influenza A virus strains of the live, attenuated FluMist vaccine (Jin *et al*, 2003). The attenuating mutations included three amino acid residues within the PB1 protein (K391E, E581G, and A661T) and one each in the PB2 (N265S) and NP (D34G) proteins. These five amino acids changes appear to be capable of conferring attenuation to other influenza A viruses, as suggested by Jin *et al* who introduced these changes into the relevant gene segments of A/Puerto Rico/8/34 (PR8) (Jin *et al*, 2004). Whereas parental PR8 replicated to high titers in ferret lung, growth of the recombinant PR8 carrying the attenuating mutations identified in A/Ann Arbor/6/60 was restricted at temperatures higher than 37°C in cell culture and was not recovered from the lungs of infected ferrets. The same approach to virus attenuation is currently being explored for producing live, attenuated influenza A H5N1 candidate vaccines (Suguitan *et al*, 2006).

As influenza viruses can cause neurological disease in humans, preclinical neurological safety testing of candidate live, attenuated influenza virus vaccines has been recommended by regulatory authorities (The European agency for the evaluation of medicinal products, 2003; International Association for Biologicals, 2006). This may be of particular importance for live H5N1-based virus vaccines, considering that avian H5N1 influenza virus strains have been associated with fatal encephalitis in humans and are highly neurovirulent in naturally and experimentally infected animals (de Jong *et al*, 2005; Shortridge *et al*, 1998; Gao *et al*, 1999; Dybing *et al*, 2000; Nishimura *et al*, 2000; Park *et al*, 2002). Preliminary data from our laboratory indicate that newborn rats intracerebrally inoculated with influenza viruses could serve as a means for assessing the neurological safety of influenza virus vaccines (Rubin *et al*, 2004).

To further test our model, we introduced five amino acid changes (K391E, E581G, and A661T change in the PB1 gene, N265S change in the PB2 gene, and D34G change in the NP gene) via site-directed mutagenesis into the respective plasmids constituting the plasmid-based reverse genetics system for A/WSN/33, a neurotoxic influenza virus strain. The rescued wild-type A/WSN/33 virus (rWSN) and the isogenic mutated A/WSN/33 virus (mu-rWSN) were then tested in rats for neurovirulence. Although rWSN and mu-rWSN replicated equally well in MDCK cells at 37°C (the body temperature of rats), in rat brain, rWSN growth continued for several days beyond the time when mu-rWSN was cleared. In addition, rWSN infection was more widespread in the brain as compared to mu-rWSN, which was restricted to cells of the choroid plexus and ventricular ependymal cells. The increased ability of rWSN to infect neurons was confirmed *in vitro* where the two viruses were incubated on primary mixed neuron-glial cultures prepared from embryonic rat brains and incubated at 37°C. These cultures, consisting of approximately 40% neurons, 50% astrocytes, and 10% microglia and other cell types were significantly more permissive to infection by rWSN than by mu-rWSN. Consistent with observations in rat brain, the predominant virus-infected cell type in these cultures was the neuron. These data suggest the possibility of use of an *in vitro* system for studying influenza virus neuroattenuation and/or for the preclinical assessment of the neurological safety of candidate influenza virus strains for humans. Moving away from animal testing in favor of alternative

in vitro methods is highly desirable. Further examination of the positive predictive value of this *in vitro* method will be the focus of future studies.

The data from our experiments described here add to the growing evidence for the possible role of the PB2 protein in virulence and are the first to suggest a possible role of the internal PB1 and NP genes on virus neuroattenuation. A Glu-to-Lys amino acid substitution at position 627 of the PB2 protein was previously reported to be associated with increased neurovirulence of an H5N1 influenza virus strain in mice (Hatta *et al*, 2001; Govorkova *et al*, 2005; Mase *et al*, 2006). This amino acid position appears be a determinant of host range and may influence virus replication efficiency (Subbarao *et al*, 1993; Shinya *et al*, 2004). The precise mechanistic role of the PB2Δ265 amino acid substitution in neuroattenuation of WSN, as well as those of PB1Δ391, PB1Δ581, PB1Δ661, and NPΔ34 remains to be determined. Importantly, however, our observations that these five loci, which were previously demonstrated to confer attenuation to both the A/Ann Arbor/6/60 and the PR8 influenza virus strains, also confer neuroattenuation to the highly neurovirulent WSN strain indicate the broad applicability of these sequences in rational vaccine design, especially for live, attenuated influenza virus strains.

Materials and methods

Viruses

An eight-plasmid DNA transfection system for the rescue of infectious influenza A virus strain A/WSN/33 from cloned cDNA was kindly provided by Dr. Webster, St. Jude Children's Research Hospital, Memphis, TN. The construction of the eight plasmids representing the PA, PB1, PB2, NS, M, NP, NA, and HA genes of the virus A/WSN/33 was described previously (Hoffmann *et al*, 2000). Three mutations in PB1 (K391E, E581G, and A661T), one in PB2 (N265S) and one in NP (D34G), identified as determinants of attenuation in other influenza virus strains (Jin *et al*, 2003, 2004), were introduced by site-directed mutagenesis into the respective cDNA plasmids. The mu-rWSN and rWSN viruses were prepared by reverse genetics as described previously (Liu and Ye, 2002). Briefly, 0.5 μg of each of the eight cDNA plasmids (pHW181-PB2 expressing the N265S mutation; pHW182-PB1 expressing the K391E, E581G, and A661T mutations; pHW185-NP expressing the D34G mutation; pHW183-PA; pHW184-HA; pHW186-NA; pHW187-M; and pHW188-NS) was incubated at room temperature for 45 min with 1 μl of TransIT LT-1 (Panvera, Madison, WI) and then placed on 293T cells grown in Opti-MEM I medium (Invitrogen, Gaithersburg, MD.) in 12-well plates. Six hours later, the DNA-transfection mixture was replaced with 0.5 ml of Opti-MEM I medium. Twenty-four hours after transfection, 0.5 ml Opti-MEM I medium containing 0.5μg of TPCK trypsin was added to the cells. At different time intervals post transfection, samples were harvested and infectious virus titer was determined by plaque assay as previously described (Rubin *et al*, 2004). Rescued rWSN or mu-rWSN was plaque purified in MDCK cells and amplified in the allantoic cavities of 9-day-old embryonated eggs. Both viruses were sequenced in their entirety to confirm genotype.

In vitro characterization of wt-WSN and mu-WSN

Confluent monolayers of MDCK cells grown in 6-well plates were infected by rWSN and mu-rWSN at a multiplicity of infection (m.o.i.) of 0.01. After 1 h of incubation at 37°C or 39°C, the inoculum was removed and 3 ml of minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) was added to each well and incubated at 37°C or 39°C. Every 12 h over a 5-day period, 0.5 ml of cell culture supernatant was collected from each plate in triplicate and replaced with 0.5 ml of fresh medium. Collected supernatants were stored at −80°C, and all were titered on the same date by plaque assay as previously described (Rubin *et al*, 2004).

Mixed cortical neuron-glial cultures were prepared from cortices of embryonic day 15 to 17 Lewis rats as previously described (Ovanesov *et al*, 2006). Briefly, meninges-free cortices were isolated, trypsinized, and mechanically dissociated by passage through fire-polished Pasteur pipettes. Washed cells were plated (3.5×10^5 cells/cm^2) onto poly-L-lysine (0.05 mg/ml)- and laminin (0.1 mg/ml)-coated glass discs in 24-well tissue culture plates and covered with 1 ml of growth medium (Dulbecco's modified Eagle's medium [DMEM]/F12 medium supplemented with 5% FBS, 5% horse serum, and a 1% penicillin-streptomycin solution; Invitrogen, Carlsbad, CA). Five days after seeding, the cell culture supernatant was removed and cells were infected by rWSN and mu-rWSN at an m.o.i. of 1.0. As controls, some wells received cell culture medium only. After 1 h of incubation at 37°C, the inoculum was removed and 1 ml of growth medium was added to each well and plates were returned to the incubator. Every 12 h over a 5-day period, 0.1 ml of cell culture supernatant was collected from each plate in triplicate and replaced with 0.1 ml of fresh growth medium. Collected supernatants were stored at −80°C and all were titered on the same date by plaque assay as previously described (Rubin *et al*, 2004).

In addition, on days 2, 4, 6, and 8 post influenza virus or mock infection, cells were immunohistochemically stained for virus protein. Briefly, cells were fixed in a 1:1 solution of acetone/methanol at 4°C for 30 min, blocked at room temperature with 3% normal goat serum for 20 min, incubated for 1 h at room temperature with a 1:400 dilution of mouse anti-influenza A virus nucleoprotein monoclonal antibody (Chemicon International, Temecula, CA) in blocking buffer, and rinsed twice in phosphate-buffered saline (PBS). Cells were then incubated

with 1.25 μg/ml of Cy3-conjugated anti-mouse immunoglobulin G (IgG) (Chemicon International) in blocking buffer for 1 h at room temperature, rinsed three times in PBS, and coverslipped with fluorescence mounting medium. For colocalization of virus antigens and cell-specific antigens, some glass disks containing fixed cells were dual stained with either (i) a 1:400 dilution of chicken anti-MAP-2 IgG (Chemicon International), followed by incubation with 2.5 μg/ml of fluorescein isothiocyanate (FITC)-conjugated anti-chicken IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for identification of neurons; or (ii) with a 1:200 dilution of rabbit anti-glial fibrillary acidic protein (GFAP) IgG (Dako, Denmark), followed by incubation with 1.25 μg/ml of Cy3-conjugated anti-rabbit IgG for identification of glial cells (astrocytes). Cells were visualized under a fluorescent confocal microscope. All antibodies were purchased from Chemicon International and diluted in blocking buffer.

In vivo *characterization of rWSN and mu-rWSN*
Multiple litters of 8 to 10 1-day-old Lewis rats (Harlan, Indianapolis, IN) were inoculated intracerebrally with 0.01 ml of MEM containing 10^4 plaque-forming units (PFU) of each of the two influenza virus strains. As a control, a litter of 10 rats was inoculated with an equivalent volume of vehicle alone. All rats were observed daily for signs of clinical disease. On each of days 1 to 6 post inoculation, three to five rats from each virus treatment group were euthanized and brains collected for virus titration. In addition, on days 1 to 6, three to five rats from each virus treatment group and two rats from the control group were euthanized and brains collected for histological and immunohistochemical examination. Strict adherence to the National Research Council's Guide for the care and use of laboratory animals was followed.

For virus titration, brains were homogenized (20% *w*/*v*) in MEM followed by brief ultrasonic disruption, and clarified by centrifugation at 2000 $\times g$ for 10 min. Aliquots of brain homogenate were stored at -80°C prior to assessment of virus concentration by plaque assay. For histology and immunohistochemistry, brains were flash-frozen in 2-methylbutane in an ethanol/dry-ice bath and then cut sagittally at midline and serially sectioned at 8 μm. The frozen sections were fixed in cold acetone and either stained with hematoxylin and eosin or with anti-influenza virus, anti-neuron and/or anti-glial cell antibodies as described above. Tissue was examined under a fluorescent confocal microscope.

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