Journal of NeuroVirology, 10: 305–314, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490499579

Taylor & Francis
Taylor & Francis Group

Wild-type and attenuated influenza virus infection of the neonatal rat brain

Steven A Rubin,[1] Dong Liu,[2] Mikhail Pletnikov,[3] Jonathan A McCullers,[4] Zhiping Ye,[1] Roland A Levandowski,[1] Jan Johannessen,[5] and Kathryn M Carbone[3,6]

[1]DVP/OVRR/CBER/FDA, Bethesda, Maryland, USA; [2]Laboratory of Neurosciences, Gerontology Research Center, NIH/NIA, Baltimore, Maryland, USA; [3]Departments of Psychiatry and Medicine, Johns Hopkins University, Baltimore, Maryland, USA; [4]St. Jude Children's Research Hospital, Memphis, Tennessee, USA; [5]OS/OC/FDA, Rockville, Maryland, USA; [6]OD/CBER/FDA Bethesda, Maryland, USA

Although influenza virus infection of humans has been associated with a wide spectrum of clinical neurological syndromes, the pathogenesis of influenza virus associated central nervous system (CNS) disease in humans remains controversial. To better study influenza virus neuropathogenesis, an animal model of influenza-associated CNS disease using human virus isolates without adaptation to an animal host was developed. This neonatal rat model of influenza virus CNS infection was developed using low-passage human isolates and shows outcomes in specific brain regions, cell types infected, and neuropathological outcomes that parallel the available literature on cases of human CNS infection. The degree of virus replication and spread in the rat brain correlated with the strains' neurotoxicity potential for humans. In addition, using sensitive neurobehavioral test paradigms, changes in brain function were found to be associated with areas of virus replication in neurons. These data suggest that further evaluation of this pathogenesis model may provide important information regarding influenza virus neuropathogenesis, and that this model may have possible utility as a preclinical assay for evaluating the neurological safety of new live attenuated influenza virus vaccine strains. *Journal of NeuroVirology* (2004) **10,** 305–314.

Keywords: influenza virus; neurotropism; neurovirulence; rat; vaccine

Introduction

Although influenza is generally regarded as an infection of the respiratory tract, neurological disease is a rare but reported complication of wild-type influenza A virus infection of humans, e.g., acute encephalitis, encephalopathy, meningitis, myelitis, and a variety of neuropathies and neuropsychiatric disorders (Takei *et al*, 1995; Morgan *et al*, 1997; Kapila *et al*, 1958; Ravenholt and Foege, 1982; Horner, 1958; Flewett and Hoult, 1958; Edelen *et al*, 1974; Togashi *et al*, 2000). Autopsy and computed tomography (CT) and magnetic resonance imaging (MRI) neuroradiological studies reveal encephalitis and multifocal symmetrical lesions in the thalamus, brain stem, cerebral periventricular white matter, and the cerebellar medulla in patients with central nervous system (CNS) disease and influenza virus infection (Protheroe and Mellor, 1991; Mizuguchi *et al*, 1995; Nagai *et al*, 1993; Kimura *et al*, 1995; Ryan *et al*, 1999; Fujimoto *et al*, 2000; Voudris *et al*, 2001). Although an etiological relationship between CNS disease in humans and influenza virus infection has not been firmly established, there is evidence that some influenza viruses can infect the human CNS. Several investigators have reported detection of influenza virus in the CNS of patients with clinically diagnosed encephalitis or encephalopathy by (1) isolation of live virus (Frankova

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

This work was supported in part by a grant from the National Vaccine Program Office and in part by an appointment to the Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. The authors thank Steve Buckingham, MD, at LeBonheur Children's Medical Center for providing the virus A/Memphis/6/01.

Received 30 December 2003; revised 23 February 2004; accepted 11 May 2004.

et al, 1977; Murphy and Hawkes, 1970; Rose and Prabhakar 1982; Thraenhart *et al*, 1975; Mori *et al*, 1999), (2) reverse transcriptase–polymerase chain reaction (RT-PCR) (Fujimoto *et al*, 1998; Takahashi *et al*, 2000; McCullers *et al*, 1999), or (3) immunohistochemical assays (Frankova *et al*, 1977).

Influenza virus virulence has been studied in several research models, including ferrets (Dowdle and Schild, 1975), mice (Ward, 1996, 1997; Lu *et al*, 1999; Mori and Kimura, 2001), rats (Ali *et al*, 1982; Jennings *et al*, 1980; Michaels *et al*, 1978; Mahmud *et al*, 1979), hamsters (Murphy *et al*, 1978; Abou-Donia *et al*, 1980), and various organ cultures (Hoke *et al*, 1979; Boudreault *et al*, 1977; Mostow *et al*, 1975). Although much has been gained from these models, most have focused on respiratory disease and/or have utilized animal-adapted virus isolates, so there is still a need to better understand the neuropathogenesis of disease caused by human (i.e., nonanimal adapted) isolates of influenza virus. Here we report the development of a neonatal rat model to study influenza virus neuropathogenesis of a variety of influenza A virus strains representing a range of apparent human neuropathogenicity, including low-passage clinical isolates. Virological, neuropathological, and neurobehavioral outcomes were examined, and showed that specific brain regions (i.e., ependyma, subventricular zone, and cerebellum) and cell types infected (predominantly neurons) and neuropathological changes (e.g., apoptosis, hydrocephalus) parallel many of the reported features of influenza virus infection of the human CNS. In addition, in the rat, the infectivity and neuroinvasiveness of the virus strains tested paralleled the strain's predicted neurotropism/neurovirulence for humans, suggesting that this model may have utility for neuropathogenesis studies of influenza virus infection of the human CNS and for providing important preclinical information about the neurotropism of new live attenuated influenza virus vaccine strains (The European Agency for the Evaluation of Medicinal Products, 2003).

Results

Groups of 30 1-day-old Lewis rats were inoculated intracerebrally with one of five different influenza virus strains: (1) A/WSN/33, a wild-type virus adapted to the rodent by serial passage in mouse brain; (2) A/Wuhan/359/95 and (3) A/Shenzhen/227/95, two attenuated, cold adapted strains; (4) A/Memphis/6/01, a clinical isolate recovered from a patient with influenza-associated encephalitis; and (5) A/Memphis/7/01, a clinical isolate recovered from a patient with respiratory disease (no CNS involvement). 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 days 2, 4, 6, 9, and 34 post inoculation (p.i.), groups of rats were euthanized and brains were removed to assess viral load, virus distribution, and neuropathology. Some rats were also tested for behavioral abnormalities.

Influenza virus infection of the neonatal rat brain

No obvious signs of disease were noted in any of the neonatally inoculated Lewis rats throughout the course of the experiment. The peak infectious virus per gram of brain was greatest in rats inoculated with A/WSN/33 or A/Memphis/6/01 (Figure 1). Peak titers of the A/Memphis/7/01 strain were significantly lower than those of A/WSN/33 ($P = .003$) and A/Memphis/6/01 ($P < .001$) at the same timepoints. Based on an assay limit of detection of 20 plaque-forming units (pfu)/ml of brain homogenate, no infectious virus could be recovered from brains of rats inoculated with the two attenuated, cold-adapted strains A/Wuhan/359/95 or A/Shenzhen/227/95. In addition, infectious virus could not be recovered from aliquots of the A/WSN/33 inocula after 24 h of incubation at 37°C in a tissue culture incubator. This indicates that any amount of virus detected in brain homogenates post infection represents *de novo* replication and not simple recovery of the original inoculum.

Virus antigen detection and distribution

Immunohistochemical staining using an anti-influenza virus nucleoprotein antibody revealed the

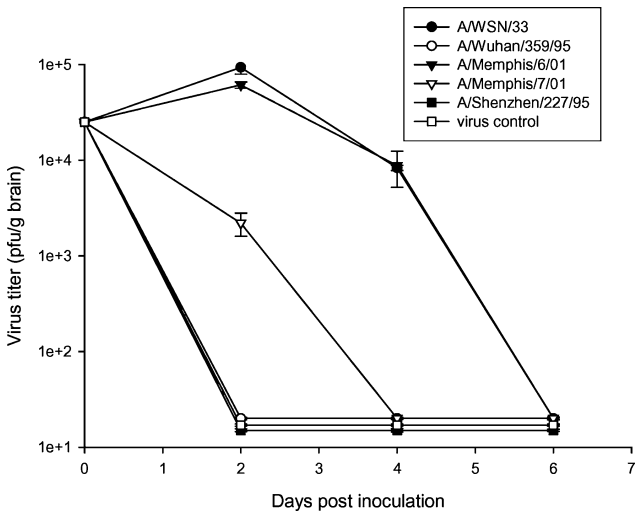

Figure 1 Infectious virus recovered from rat brains inoculated with A/Wuhan/359/95 (*open circles*), A/Shenzhen/227/95 (*solid squares*), A/Memphis/7/01 (*open triangle*), A/Memphis/6/01 (*solid triangle*), and A/WSN/33 (*solid circles*). Each time point represents an average of three to five rat brains. A/WSN/33 incubated at 37°C in a tissue culture incubator (*open squares*) served as a control for virus replication. Error bars represent the standard error of the mean.

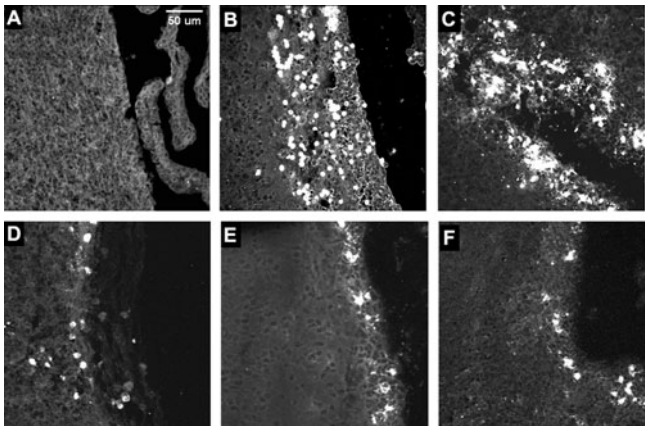

Figure 2 Sections of rat brain immunohistochemically stained with anti–influenza A virus monoclonal antibody showing fluorescent signal indicating periventricular virus antigen expression on day 3 p.i. in rat brain inoculated with (**A**) uninfected material, (**B**) A/WSN/33, (**C**) A/Memphis/6/01, (**D**) A/Memphis/7/01, (**E**) A/Shenzhen/227/95, and (**F**) A/Wuhan/359/95. Note the relatively extensive infection in rats inoculated with virus strains associated with CNS disease (**B** and **C**) compared to the limited infection seen in rats inoculated with those not associated with CNS disease (**D**–**F**).

presence of virus in the choroid plexus and periventricular area of the lateral and third ventricles in all virus inoculated rats, even in cases where infectious virus could not be recovered, e.g., A/Wuhan/359/95 or A/Shenzhen/227/95. In all groups, the intensity of the staining was greatest at day 2 p.i., gradually declining by day 6 p.i., and undetectable at or beyond 9 days p.i. The extent of virus spread in the brain was strain dependent (Figure 2). In rats inoculated with the two cold-adapted, attenuated laboratory strains, virus infection was restricted to a few ventricular ependymal cells lining the lateral ventricle. In rats inoculated with the clinical isolate A/Memphis/7/01, a greater number of ependymal cells were infected and a few scattered virus-infected cells were also seen deeper in brain parenchyma surrounding the lateral ventricle. In rats inoculated with the neurovirulent A/WSN/33 and A/Memphis/6/01 strains, infection was more widespread, with numerous virus-infected cells in the ependyma, meninges, underlying neurons in the dorsal lateral subventricular zone, corpus callosum, and external granule and molecular layers of the cerebellum (Figure 3). Double labeling with cell-specific antibodies to the astrocyte glial fibrillary acidic protein (GFAP) or to the neuronal microtubule-associated protein (MAP) demonstrated that most of the infected cells were neurons (Figure 4). Infected astrocytes were seen rarely, usually in areas that contained many infected neurons.

Apoptosis

Significant concentrations of terminal deoxynucleotidyltransferase-mediated dUTp nick-end labeling (TUNEL)-positive cells, suggesting cell apoptosis, were detected in virus-infected brain regions, in both neurons and glial cells as demonstrated by double labeling with antibodies to neuron-specific enolase (NSE) or GFAP, respectively (data not shown). Interestingly, many apoptotic cells were not virus infected (Figure 5). A few scattered TUNEL-positive cells were also seen in uninfected brain regions as well as in the control rats and are believed to be cells undergoing apoptosis as part of the normal brain development process (Martin, 2001; Sastry and Rao, 2000). This is in contrast to the numerous TUNEL-positive cells concentrated in infected brain regions.

Neuropathology

The only gross neuropathological change seen in influenza virus-infected rats was enlargement of the lateral ventricle (hydrocephalus) and edema as detected by MRI (Figure 6). This was observed in five (63%) out of the eight A/WSN/33-inoculated rats and in four (67%) of the six A/Memphis/6/01-inoculated rat brains. The severity of hydrocephalus was similar between the A/WSN/33-inoculated rats and the A/Memphis/6/01-inoculated-rats. The mean ratio of the ventricular volume to the whole brain volume in untreated control rats was 0.007 (or 0.7%), whereas that in the hydrocephalic A/WSN/33- and A/Memphis/6/01-inoculated rats was 4.7 ($P = .01$) and 6.7-fold greater ($P = .008$), respectively. None of the rats inoculated with the other influenza virus strains developed hydrocephalus.

Based on hematoxylin and eosin (H&E) staining of brain sections of rats inoculated with any of the five

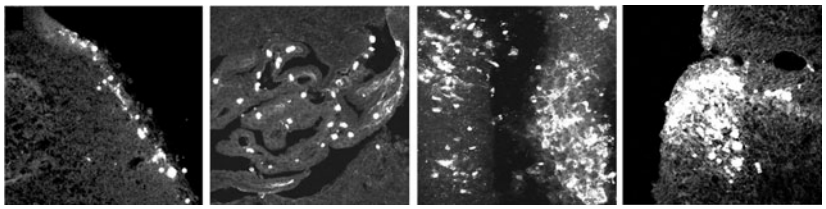

Figure 3 Distribution of viral antigen in rat brain. Sections of rat brain immunohistochemically stained with anti–influenza A virus monoclonal antibody showing fluorescent signal indicating virus antigen expression on day 3 p.i. in the (**A**) meninges (A/WSN/33), (**B**) choroid plexus (A/Memphis/6/01), (**C**) subventricular zone (A/Memphis/6/01), and (**D**) external granule cell and molecular layers of the cerebellum (A/WSN/33).

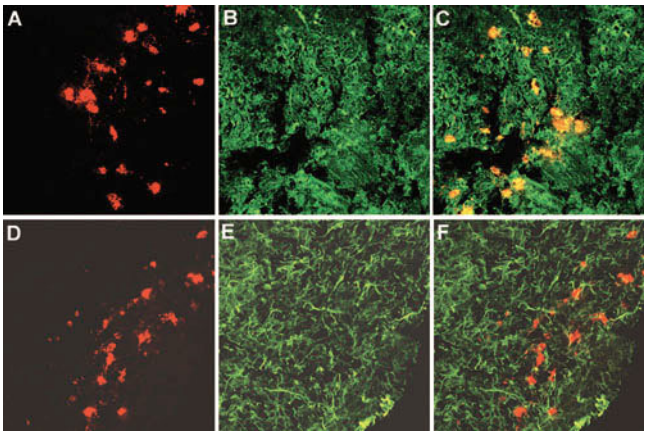

Figure 4 Confocal laser scanning microscopy of the subventricular region of a rat infected with A/Memphis/6/01 showing colocalization of virus antigen with neurons and not astrocytes on day 3 p.i. **A** and **D**, A/Memphis/6/01 infected cells (*red*). **B**, MAP expressing neuronal cells (*green*). **C**, Coincident confocal image of **A** and **B** demonstrating virus antigen expression in neuronal cells (*yellow*). **E**, GFAP expressing astrocytes (*green*). **F**, Coincident confocal image of **D** and **E** demonstrating the absence of virus antigen expression in astrocytes.

virus strains, there was no evidence of inflammatory cell infiltration in the brain, including meningitis, ventriculitis, or encephalitis, at any timepoint (days 2, 4, 6, 9, and 34 p.i.).

Functional neurological abnormalities: neurobehavioral testing

Because the A/WSN/33 and A/Memphis/6/01 influenza virus strains were found to heavily infect the brain during a time of critical neurodevelopmental activity, sensitive neurobehavioral testing was performed to examine the rats for functional neurological damage.

Rats inoculated with the A/Memphis/6/01 influenza virus strain exhibited significantly greater novelty-induced horizontal activity (NIHA) compared to control rats. Although A/WSN/33-infected rats exhibited a moderate increase in NIHA relative to control rats, these differences were not statistically significant (Figure 7A). An analysis of the data for NIHA showed a significant effect of the infection status, $F(2, 39) = 5.3$, $P = .01$, with no interaction

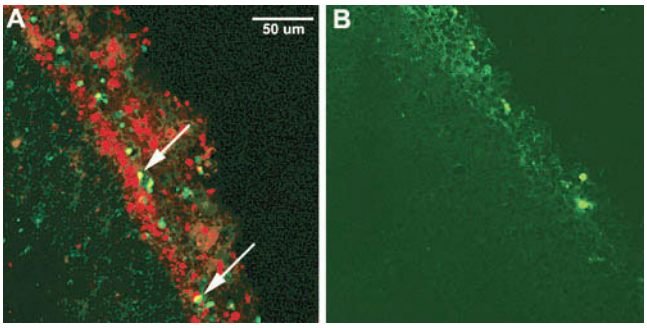

Figure 5 Confocal laser scanning microscopy of the periventricular region of a rat 4 days post inoculation with (**A**) A/WSN/33 or (**B**) uninfected material showing that in the A/WSN/33-infected rat, many apoptotic cells (*green*) are not virus infected (*red*). A few cells (*arrows*) express both markers (*yellow*), indicating apoptotic virus-infected cells. Note expression of a limited number of apoptotic cells (*green*) in uninfected rat brain section indicating normal levels of apoptosis.

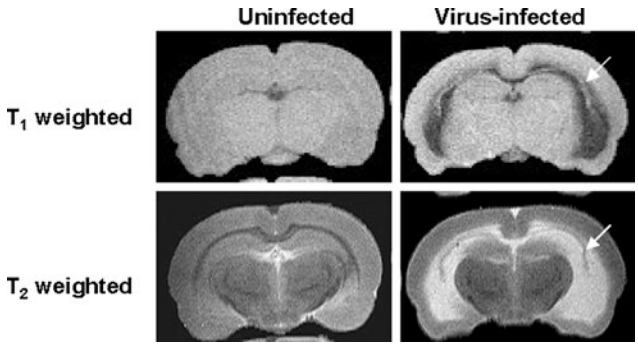

Figure 6 T_1- (*upper row*) and T_2-weighted (*lower row*) MRI images through the hippocampus of a formalin-fixed control brain (*left*) and a formalin-fixed A/WSN/33-inoculated brain. T_1-weighted images provide little white-gray matter contrast, but clearly show enlarged ventricles in virus-infected brain. In the T_2-weighted images, ventricular fluid appears bright, as do areas of periventricular edema.

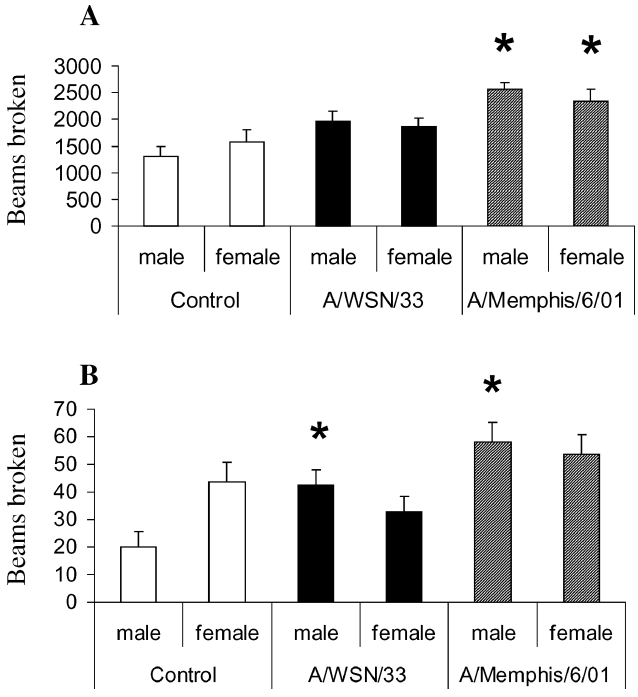

Figure 7 Effects of neonatal flu infection on novelty-induced activity in Lewis rats on PND 30. Novelty-induced horizontal (**A**) and vertical (**B**) activity in control (*open bars*), A/WSN/33-infected (*solid bars*), and A/Memphis/6/01-infected (*striped bars*). Note a greater novelty-induced horizontal activity in the male and female Memphis-infected rats compared to the male and female control rats in the **A**, and significantly higher novelty induced vertical activity in the male A/WSN/33-infected and Memphis-infected rats compared to male control rats in **B**. $^*P < .05$ versus the control rats of the same sex group. Error bars represent the standard error of the mean.

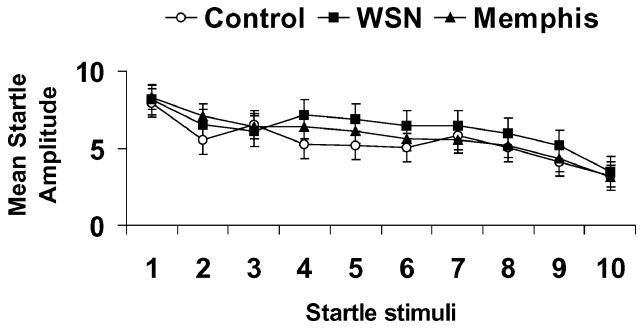

Figure 8 Effects of neonatal flu infection on habituation of the acoustic startle response in Lewis rats on PND 30. Note a comparable habituation of the acoustic startle in the rats of all groups, including uninfected control animals. Error bars represent the standard error of the mean.

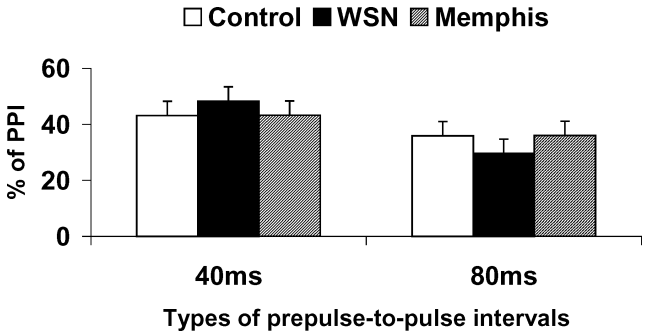

Figure 9 Effects of neonatal flu infection on prepulse inhibition of the acoustic startle in Lewis rats on PND 30. Note the similar prepulse inhibition of the acoustic startle in the rats of all groups, including uninfected animals. Error bars represent the standard error of the mean.

between infection status and sex, $P > .05$. The P values obtained from post hoc comparison testing (Tukey test) for A/Memphis/6/01- and A/WSN/33-infected rats versus control rats were .01 and .05, respectively.

Rats inoculated with either A/Memphis/6/01 or A/WSN/33 showed increased novelty-induced vertical activity (NIVA) compared to control rats. Interestingly, the virus-induced elevation of NIVA was only observed in male rats; female rats showed no differences compared to control female rats (Figure 7B). An analysis of the data for NIVA revealed significant effects of infection status, $F(2, 39) = 6.5$, $P = .004$, and the infection status by sex interaction, $F(2, 39) = 3.96$, $P = .028$. Post hoc comparison testing confirmed that male rats from both the A/WSN/33-infected group and the A/Memphis/6/01-infected group exhibited greater NIVA relative to control animals ($P = .025$ and $P < .001$, respectively). Notably, sex differences in behavioral tests are common.

Neonatal infection of the rat brain with the A/Memphis/6/01 and A/WSN/33 influenza virus strains did not induce alteration in startle responsiveness or habituation of the acoustic startle response (Figure 8). Likewise, there were no alterations in repulse inhibition of the acoustic startle response in A/Memphis/6/01- or A/WSN/33-infected rats compared to control animals (Figure 9).

Discussion

Experimental influenza virus infection of the rat brain has not been previously described. Here, five influenza virus strains covering a range of human neurovirulence potential were directly inoculated into rat brain and outcomes were assessed. The infectivity and neuroinvasiveness of the five virus strains in rat brain paralleled their predicted neurovirulence for humans, and, based upon the data can be divided into three general groups: (1) a "low"-neurovirulence group including the two attenuated strains A/Wuhan/359/95 and A/Shenzhen/227/95, (2) an "intermediate"-neurovirulence group including the wild-type strain A/Memphis/7/01, and (3) a "high"-neurovirulence group including the A/WSN/33 and A/Memphis/6/01 strains. Outcomes of infection of the rat brain by these viruses are summarized in Table 1.

Evidence that influenza virus strains can replicate in rat brain was provided by the recovery of A/WSN/33, A/Memphis/6/01, and A/Memphis/7/01 at or beyond day 2 post inoculation. In some cases, the recovered titers exceed that of the inocula. Infectious virus could not be recovered from aliquots of the A/WSN/33 inocula after 24 h of incubation at 37°C in a tissue culture incubator, indicating that virus detected in brain homogenates post infection

Table 1 Summary of outcomes of infection in rats by influenza virus strains of varying neurovirulence potential

Virus group	*Group members*	*Replication in brain*	*Distribution in brain*	*Pathology*	*Behavioral abnormalities*
Low NV	A/Wuhan/359/95 A/Shenzhen/227/95	–	CP, EP	None detected	Not tested
Intermediate NV	A/Memphis/7/01	+	CP, EP, PV	None detected	Not tested
High NV	A/WSN/33 A/Memphis/6/01	++	CP, EP, PV, SVZ, M, CC, CB	hydrocephalus, apoptosis	↑NIVA ↑NIHA

Abbreviations: NV—neurovirulence; CP—choroid plexus; EP—ventricular ependymal cell lining; PV—periventricular cells; SVZ—subventricular zone; M—meninges; CC—corpus callosum; CB—cerebellum; NIVA—novelty-induced vertical activity; NIHA—novelty-induced horizontal activity.
(–) incomplete or no replication; (+) limited replication; (++) productive replication.

represents *de novo* replication and not simple recovery of the original inoculum. It should be noted that although influenza virus was found to productively replicate in rat brain, the level of replication was relatively low. Influenza viruses, as unnatural agents of infection in rats, certainly do not replicate in the brain to the same extent that can be seen following natural infection by other viruses whereupon multiple log increases in virus concentration can be observed.

Additional evidence for productive infection of the brain is provided by immunohistochemically stained sections of rat brains inoculated with members of the "high"-neurovirulence group. Here, cell-to-cell spread of virus and dissemination to the meninges, cerebellum, dorsal lateral subventricular zone, and the corpus callosum is clear. Virus spread likely occurs via circulation of virus through the cerebral spinal fluid (CSF); however, infection of cells deeper in the brain parenchyma may indicate intraneuronal spread via retrograde axonal transport from the infected cells lining the ventricles (Mori and Kimura, 2001). Associated with infection of by virus, some rats developed hydrocephalus, parenchymal edema, and apoptosis. No other pathology was noted.

In contrast to the members of the "high"-neurovirulence group, those of the "low"-neurovirulence group could not be recovered at any timepoint and immunohistochemistry showed limited cell-to-cell spread with transient, focal, and, perhaps incomplete replication. Of note, this does not appear to be due to an inability of these two cold-adapted viruses to replicate at 37°C (the temperature of rat brain), because, at this temperature in *in vitro* cell culture (MDCK cells), both viruses replicated to equivalent peak titers with similar kinetics as compared to A/WSN/33, A/Memphis/6/01 and A/Memphis/7/01 (data not shown).

The wild-type A/Memphis/7/01 strain, isolated from patient withouth CNS symptoms, was able to replicate and spread in the brain to a greater extent than the attenuated strains but not to the level attained by the neurovirulent strains.

Importantly, cell-specific targets of influenza virus in rats (neurons within the subventricular zone and the cerebellum) and associated neuropathology (hydrocephalus and apoptosis) have also been observed in humans (Frankova *et al*, 1977; Takahashi *et al*, 2000; Conover and Roessmann, 1990; Sarnat *et al*, 1979; Nakai *et al*, 2003). Thus, the rat model of influenza virus CNS infection may be applicable for the study of influenza virus CNS infection of humans. One observation in rats that may contribute to our understanding of influenza virus infection of the human brain is provided by our apoptosis study, which showed that many of the apoptotic cells were not virus infected. This indicates release of cytokines and other soluble factors from infected cells as an indirect mechanism of death of neighboring cells, as has been reported in other infections (Kaul *et al*, 2001). Indeed, induction of cytokines and other soluble factors have been reported in cases of influenza virus encephalopathy and encephalitis (Aiba *et al*, 2001; Yokota *et al*, 2000).

Because A/Memphis/6/01 and A/WSN/33 were found to infect brain structures that mature postnatally (dorsal lateral subventricular zone and cerebellum) and are known to be involved in inhibitory control of sensory and motor functions (Ito, 1989; Swerdlow *et al*, 2001), functional correlates of infection were investigated. It was postulated that virus infection of these brain areas might be associated with behavioral disinhibition, manifested as elevated locomotor activity in response to novel environmental stimuli. Neurobehavioral tests performed on adult rats neonatally inoculated with these viruses showed an increase in NIHA (A/Memphis/6/01) and NIVA (A/Memphis/6/01 and A/WSN/33). In contrast, no abnormalities were found in other neurobehavioral tests, such as prepulse inhibition and habituation of the acoustic startle response, which are behaviors largely controlled by areas of the brain fully matured at the time of inoculation. The histological and behavioral data suggest that areas of ongoing brain development are sensitive to influenza virus infection and associated damage, whereas areas fully matured prenatally (i.e., prior to influenza virus inoculation) are relatively spared from infection and functional damage. In addition, these results indicate that the neurological damage from early virus infection appears to be persistent, as the observed behavioral abnormalities were measured long after virus replication could no longer be detected in the brain. Notably, influenza virus–associated neurobehavioral abnormalities also have been reported in mice; however, these were found to be a result of the maternal immune response on the fetus rather than direct infection (Shi *et al*, 2003). Our rat model suggests neurobehavioral abnormalities can also result from direct and focal influenza virus infection of the rat CNS. Both of these rodent models deserve further study and may hold promise for providing more insight into the highly controversial reported associations between *in utero* exposure to influenza virus and risk for subsequent developmental CNS damage in humans (Munk-Jorgensen and Ewald, 2001; de Messias *et al*, 2001; Takei *et al*, 1995, 1996; Adams *et al*, 1993; Livingston *et al*, 1993).

The parallels between outcomes of influenza virus infection of the neonatal rat and of the human brain (e.g., infected cell type, regional distribution, neuropathology, and possibly behavioral abnormalities) support the value of this rat model in the study of influenza virus neuropathogensis of the human. In addition, the finding that the extent of virus growth and spread in neonatal rat brain and the microscopic and macroscopic degree of neuropathological damage was able to discriminate between strains similar to current live, attenuated influenza vaccine strains, i.e., "low"-neurovirulence strains and wild-type, low-passage human isolates (e.g., representing

"intermediate"- and "high"-neurovirulence strains), suggests that this model may also be useful in obtaining preclinical information about the neurotropism of new strains of live attenuated influenza virus vaccines, without need for prior adaptation to the rodent. Indeed, although neurovirulence testing of live, attenuated influenza virus vaccines has been recommended (The European Agency for the Evaluation of Medicinal Products, 2003; European Pharmacopeia Comission, 1997), currently there is no such test available. With additional validation (e.g., testing of additional virus strains), it is possible that this model may have a contribution to make to the regulation of live, attenuated influenza virus vaccines.

Materials and methods

Rats and viruses

Groups of 30 1-day-old Lewis rats (Harlan, Indianapolis, IN) were inoculated intracerebrally with 0.01 ml of minimal essential media (MEM) containing 10^4 pfu of one of several influenza virus strains: (1) A/WSN/33 (H1N1), a wild-type virus adapted to the rodent by serial passage in mouse brain (Francis and Moore, 1940) and expanded by passage in specific pathogen-free hens eggs; (2) A/Wuhan/359/95, an attenuated, cold-adapted H3N2 strain passaged in specific pathogen–free hens eggs; (3) A/Shenzhen/227/95, an attenuated, cold-adapted H1N1 strain passaged in specific pathogen–free hens eggs; (4) A/Memphis/6/01 (H1N1), a clinical isolate recovered from a nasopharyngeal swab from a patient with influenza-associated encephalitis, passaged twice in Madin-Darby canine kidney (MDCK) cells; and (5) A/Memphis/7/01 (H1N1), a clinical isolate recovered from a nasopharyngeal swab from a patient with respiratory disease (no CNS involvement), passaged twice in MDCK cells. All viruses underwent one additional passage in MDCK cells prior to inoculating into rats. 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 (lethargy, ataxia, paresis) and were weighed every other day throughout a 3-week period to assess general health. Some rats were tested for behavioral abnormalities on postnatal day 30. On days 2, 4, 6, 9, and 34 post inoculation (p.i.), rats were anesthetized, exsanguinated, and perfused through the heart with 0.01 M phosphate-buffered saline (PBS). Strict adherence to the National Research Council *Guide for the Care and Use of Laboratory Animals* was followed.

Virus isolation and titration

The brain was removed from three to five rats from each virus-inoculated group and from one rat from the control group at each time point. The brain was homogenized (20% *w/v*) in MEM containing 2% bovine serum albumin (BSA), 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. To discriminate between recovery of virus that had underwent replication in the brain from recovery of the original inoculum, three aliquots of the A/WSN/33 strain were diluted in MEM to a concentration of 1×10^6 pfu/ml (a concentration equivalent to the inocula) and placed 37°C in a tissue culture incubator. On days 0, 2, 4, and 6 post incubation, 100-μl volumes were removed from these preparations stored at -80°C prior to assessment of virus concentration by plaque assay.

Virus titer in brain homogenates and in virus stocks was determined by making 10-fold serial dilutions of the material in MEM containing 4% BSA and 1.0 μg/ml of TPCK-treated trypsin (Worthington Biochemical, Freehold, NJ) and incubating for one hour at 35°C on MDCK cell monolayers. Viral inoculum was removed and cell monolayers were rinsed with MEM before covering with 0.5% Noble agar in 2× MEM (Quality Biologicals, Gaithersburg, MD) supplemented with 4% BSA and 1 μg/ml of TPCK-treated trypsin. After 3 days of incubation at 35°C, the agar overlay was removed and monolayers were fixed in methanol for 30 min. Plaques were visualized by 1% cresyl violet staining. Determination of number of plaques per ml of brain homogenate was used to calculate infectious virus titer/gram of brain. The limit of detection for this assay is 20 pfu per ml of brain homogenate or cell culture supernatant.

Histology and immunohistochemistry

At each time point, three rats from each virus-infected group and one rat from the control group underwent intracardiac perfusion with PBS followed by 4% paraformaldehyde. Brain was removed and postfixed in 4% paraformaldehyde overnight at 4°C and then flash-frozen in 2-methylbutane in an ethanol/dry-ice bath. Brains were cut sagittally at midline and serially sectioned at 8 μm. For histopathological evaluation, brain sections were stained with H&E. For immunohistochemical analysis, brain sections were fixed in acetone, blocked with 3% normal goat serum in PBS, and incubated with mouse anti-influenza A virus nucleoprotein monoclonal antibody (Chemicon International Inc., Temecula, CA.). For colocalization of virus antigens and cell-specific antigens, some brain sections were dual stained with rabbit anti-GFAP polyclonal antibody (Dako Corp., Carpenteria, CA) for identification of astrocytes or with rabbit anti–MAP-2 or NSE (Chemicon International Inc.) for identification of neurons. Following the addition of Cy3-conjugated anti-mouse immunoglobulin G (IgG) and/or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibodies (Chemicon International Inc.), tissue was visualized under a fluorescent confocal microscope.

Magnetic resonance imaging methods and analysis
Thirty days after influenza virus infection, MRI using a Bruker 2T MRI machine with custom-made rat coil was used to assess neuropathology in eight A/WSN/33-infected and six A/Memphis/6/01-infected rat brains. Coronal T_1- and T_2-weighted images were acquired from whole brain with TE 52.6 ms, TM 29.2 ms, big delta 50 ms, and b values of 300 and 30,000 s/cm^2.

Detection of apoptosis
Some sections of brain tissue stained with anti–influenza A virus monoclonal antibody were dual labeled for DNA fragmentation using a modification of the TUNEL method (Trevigen, Inc., Gaithersburg, MD). Briefly, after acetone treatment, tissue was incubated with dNTP, biotinylated dUTP, and terminal transferase (TdT) in a humidified chamber at 37°C for 1 h. The reaction was stopped by immersing the slides in 0.1 M NaCl in 300 mM sodium citrate. The conjugates were amplified with streptavidin–horseradish peroxidase and visualized with streptavidin-FITC. DNase1-treated tissue was used as positive control and omission of DNA polymerase was used as a negative control. Some brain sections were dual stained as described above for identification of astrocytes or neurons.

Neurobehavioral experiments
Novelty-induced activity: Novelty-induced activity was assessed in rats inoculated with the A/WSN/33 ($n = 8$ males and 9 females) and A/Memphis/6/01 ($n = 5$ males and 5 females) influenza virus strains. As controls, five male and eight female rats inoculated with vehicle alone were assessed. Activity chambers (San Diego Instruments, San Diego, CA) were used to measure NIHA and NIVA over a 30-min period. Both types of the locomotion were automatically recorded with the software and later analyzed.

Acoustic startle response (ASR): Startle chambers (SDI, San Diego, CA) were used for measuring startle reactivity and plasticity. Each rat was placed in a Plexiglas cylinder (9 cm in diameter) within each chamber. A loudspeaker mounted 24 cm above the cylinder provided broadband background noise and the acoustic stimuli. Presentations of the acoustic stimuli were controlled by SR-LAB software (SDI, San Diego, CA) and interface system, which also rectified, digitized, and recorded responses from the accelerometer. The maximum voltages within 50-ms reading windows, starting at stimulus onset, were used as the measures of startle amplitudes. Sound levels were measured inside the startle cabinets by means of the digital sound level meter (Realistic, Tandy Corporation, Fort Worth, TX). The accelerometer sensitivities within each startle chamber were calibrated regularly and were found to remain constant over the test period.

Acoustic startle response was assessed in rats inoculated with the A/WSN/33 ($n = 8$ males and 9 females) and A/Memphis/6/01 ($n = 5$ males and 5 females) influenza virus strains. As controls, five male and eight female rats inoculated with vehicle alone were assessed. The experimental session consisted of a 5-min acclimatization period to a 65-dB background noise (continuous throughout the session), followed by the presentation of 10 100-ms 108-dB white noise stimuli at a 20-s interstimulus interval (the habituation session). Amplitudes of the ASR during the habituation session were analyzed and presented as the maximum value of the startle response in relation to the rat's body weight. These weight-corrected ASRs were determined by dividing the ASR value by the weight in grams of the test subject.

Upon the completion of the habituation session, each rat was tested in the prepulse inhibition (PPI) session. During each PPI session, a rat was exposed to the following types of trials: pulse-alone trial (a 108-dB, 100-ms, broadband burst); the omission of stimuli (no-stimulus trial); and two prepulse-pulse combinations (prepulse-pulse trials). A 50-ms broadband burst was used as a prepulse. Prepulse-pulse combinations included the prepulse intensity (10 dB above the background noise) and two prepulse-to-pulse intervals (40 and 80 ms). Each session consisted of eight pulse-alone trials, six of each prepulse-pulse trials (combinations), and six no-stimulus trials. All trials were presented in pseudorandom order. PPI was assessed as the percentage scores of PPI (%PPI): 100 × (mean startle amplitude on pulse-alone trials − mean startle amplitude on prepulse-pulse trials/mean startle amplitude on pulse-alone trials) for each animal separately. The percentage of PPI for each animal was used the dependent variable in the statistical analysis.

Statistical analysis
The data are presented as mean ± SEM. Two-way analysis of variance (ANOVA) was used to analyze the data for NIHA and NIVA with infection status and sex as independent variables. Two-way repeated-measures ANOVA were utilized to analyze the data from the habituation of the acoustic startle experiments with infection status and stimuli (a repeated measure) as independent variables. Two-way ANOVA was used to analyze the data for the prepulse inhibition experiments with infection status and type of prepulse as independent variables. Tukey tests for multiple comparisons were used when applicable. When the data did not pass the normality test and/or equal variance test, the data were subjected to the rank transformation, and ANOVAs were rerun on the transformed data. A $P < .05$ was considered as the criterion for statistical significance.

References

Abou-Donia H, Jennings R, Potter CW (1980). Growth of influenza A viruses in hamsters. *Arch Virol* **65:** 99–107.

Adams W, Kendell RE, Hare EH, Munk-Jorgensen P (1993). Epidemiological evidence that maternal influenza contributes to the aetiology of schizophrenia. An analysis of Scottish, English, and Danish data. *Br J Psychiatry* **163:** 522–534.

Aiba H, Mochizuki M, Kimura M, Hojo H (2001). Predictive value of serum interleukin-6 level in influenza virus-associated encephalopathy. *Neurology* **57:** 295–299.

Ali M, Maassab H, Jennings R, Potter C (1982). Infant rat model of attenuation for recombinant influenza viruses prepared from cold-adapted attenuated A/Ann Arbor/6/60. *Infect Immun* **38:** 610–619.

Boudreault A, Diaz-Rodriguez P, Lecomte J (1977). In vitro markers for measuring residual virulence for men of live attenuated influenza viruses. *Dev Biol Stand* **39:** 33–40.

Conover PT, Roessmann U (1990). Malformational complex in an infant with intrauterine influenza viral infection. *Arch Pathol Lab Med* **114:** 535–538.

de Messias EL, Cordeiro NF, Sampaio JJ, Bartko JJ, Kirkpatrick B (2001). Schizophrenia and season of birth in a tropical region: relationship to rainfall. *Schizophr Res* **48:** 227–234.

Dowdle WR, Schild GC (1975). Laboratory propagation of human influenza viruses, experimental host range, and isolation from clinical material. In *The influenza viruses and influenza.* Kilbourne E (ed). New York: Academic Press, pp 243–268.

Edelen J, Bender T, Chin T (1974). Encephalopathy and pericarditis during an outbreak of influenza. *Am J Epidemol* **100:** 79–84.

European Pharmacopeia Comission (1997). Tests for neurovirulence of live virus vaccines (general method no. 2.6.18). In *European pharmacopeia*, p 99.

Flewett T, Hoult J (1958). Influenzal encephalopathy and post-influenzal encephalitis. *Lancet* **ii:** 11–15.

Francis T, Moore A (1940). A study on the neurotropic tendency in strains of the virus of epidemic influenza. *J Exp Med* **72:** 717–728.

Frankova V, Jirasek A, Tumov B (1977). Type A influenza: postmortem virus isolations from different organs in human lethal cases. *Arch Virol* **53:** 265–268.

Fujimoto S, Kobayashi M, Uemura O, Iwasa M, Ando T, Katoh T, Nakamura C, Maki N, Togari H, Wada Y (1998). PCR on cerebral spinal fluid to show influenza-associated acute encephalopathy or encephalitis. *Lancet* **352:** 873–875.

Fujimoto Y, Shibata M, Tsuyuki M, Okada M, Tsuzuki K (2000). Influenza A virus encephalopathy with symmetrical thalamic lesions. *Eur J Pediatr* **159:** 319–321.

Hoke CH Jr, Hopkins JA, Meiklejohn G, Mostow SR (1979). Comparison of several wild-type influenza viruses in the ferret tracheal organ culture system. *Rev Infect Dis* **1:** 946–954.

Horner F (1958). Neurologic disorders after Asian influenza. *N Engl J Med* **258:** 983–985.

Ito M (1989). Long-term depression. *Annu Rev Neurosci* **12:** 85–102.

Jennings R, Potter CW, Teh CZ, Mahmud MI (1980). The replication of type A influenza viruses in the infant rat: a marker for virus attenuation. *J Gen Virol* **49:** 343–354.

Kapila C, Kaul S, Kalayana T, Bannerjee D (1958). Neurological hepatic disorders associated with influenza. *BMJ* **2:** 1311–1314.

Kaul M, Garden GA, Lipton SA (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410:** 988–994.

Kimura S, Kobayashi T, Osaka H, Shimizu C, Uehara S, Ohtuki N (1995). Serial magnetic resonance imaging in post-infectious focal encephalitis due to influenza virus. *J Neurol Sci* **131:** 74–77.

Livingston R, Adam BS, Bracha HS (1993). Season of birth and neurodevelopmental disorders: summer birth is associated with dyslexia. *J Am Acad Child Adolesc Psychiatry* **32:** 612–616.

Lu X, Tumpey T, Morken T, Zaki S, Cox N, Katz J (1999). A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J Virol* **73:** 5903–5911.

Mahmud MI, Jennings R, Potter CW (1979). The infant rat as a model for assessment of the attenuation of human influenza viruses. *J Med Microbiol* **12:** 43–54.

Martin LJ (2001). Neuronal cell death in nervous system development, disease, and injury (review). *Int J Mol Med* **7:** 455–478.

McCullers JA, Facchini S, Chesney PJ, Webster RG (1999). Influenza B virus encephalitis. *Clin Infect Dis* **28:** 898–900.

Michaels RH, Mahmud MI, Coup AJ, Jennings R, Potter CW (1978). Influenza virus infection in newborn rats: a possible marker of attenuation for man. *J Med Virol* **2:** 253–264.

Mizuguchi M, Abe J, Mikkaichi K, Yoshida K, Yamanaka T, Kamoshita S (1995). Acute necrotizing encephalopathy of childhood: a new syndrome presenting with multifocal, symmetric brain lesions. *J Neurol Neurosurg Psychiatry* **58:** 555–561.

Morgan V, Castle D, Page A, Fazio S, Gurrin L, Burton P, Montgomery P, Jablensky A (1997). Influenza epidemics and incidence of schizophrenia, affective disorders and mental retardation in Western Australia: no evidence of a major effect. *Schizophr Res* **26:** 25–39.

Mori I, Kimura Y (2001). Neuropathogenesis of influenza virus infection in mice. *Microbes Infect* **3:** 475–479.

Mori S, Nagashima M, Sasaki Y, Mori K, Tabei Y, Yoshida Y, Yamazaki K, Hirata I, Ito T, Suzuki S (1999). A novel amino acid substitution at the receptor-binding site on the hemagglutinin of H3N2 influenza A viruses isolated from 6 cases with acute encephalopathy during the 1997–1998 season in Tokyo. *Arch Virol* **144:** 147–155.

Mostow SR, Flatauer S, Martinko J (1975). An 'in vitro' marker of attenuation for live influenza virus vaccine candidates. *Dev Biol Stand* **28:** 354–362.

Munk-Jorgensen P, Ewald H (2001). Epidemiology in neurobiological research: exemplified by the influenza-schizophrenia theory. *Br J Psychiatry Suppl* **40:** s30–s32.

Murphy A, Hawkes R (1970). Neurological complications of influenza A2/Hong Kong/68 virus. *Med J Aust* **2:** 511.

Murphy BR, Wood FT, Massicot JG, Chanock RM (1978). Temperature-sensitive mutants of influenza virus. XVI. Transfer of the two ts lesions present in the Udorn/

72-ts-1A2 donor virus to the Victoria/3/75 wild-type virus. *Virology* **88:** 244–251.

Nagai T, Yagishita A, Tsuchiya Y, Asamura S, Kurokawa H, Matsou N (1993). Symmetrical thalamic lesions on CT in influenza A virus infection presenting with or without Reye syndrome. *Brain Dev* **15:** 67–74.

Nakai Y, Itoh M, Mizuguchi M, Ozawa H, Okazaki E, Kobayashi Y, Takahashi M, Ohtani K, Ogawa A, Narita M, Togashi T, Takashima S (2003). Apoptosis and microglial activation in influenza encephalopathy. *Acta Neuropathol (Berl)* **105:** 233–239.

Protheroe S, Mellor D (1991). Imaging in influenza A encephalitis. *Arch Dis Child* **66:** 702–705.

Ravenholt R, Foege W (1982). 1918 influenza, encephalitis lethargica, Parkinsonism. *Lancet* **2:** 860–864.

Rose E, Prabhakar P (1982). Influenza A virus associated neurological disorders in Jamaica. *WI Med J* **31:** 29–33.

Ryan MM, Procopis PG, Ouvrier RA (1999). Influenza A encephalitis with movement disorder. *Pediatr Neurol* **21:** 669–673.

Sarnat HB, Rybak G, Kotagal S, Blair JD (1979). Cerebral embryopathy in late first trimester: possible association with swine influenza vaccine. *Teratology* **20:** 93–99.

Sastry PS, Rao KS (2000). Apoptosis and the nervous system. *J Neurochem* **74:** 1–20.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003). Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *J Neurosci* **23:** 297–302.

Swerdlow NR, Halim N, Hanlon FM, Platten A, Auerbach PP (2001). Lesion size and amphetamine hyperlocomotion after neonatal ventral hippocampal lesions: more is less. *Brain Res Bull* **55:** 71–77.

Takahashi M, Yamada T, Nakashita Y, Saikusa H, Deguchi M, Kida H, Tashiro M, Toyoda T (2000). Influenza virus-induced encephalopathy: clinicopathologic study of an autopsied case. *Pediatr Int* **42:** 204–214.

Takei N, Mortensen PB, Klaening U, Murray RM, Sham PC, O'Callaghan E, Munk-Jorgensen P (1996). Relationship between in utero exposure to influenza epidemics and risk of schizophrenia in Denmark. *Biol Psychiatry* **40:** 817–824.

Takei N, Murray G, O'Callaghan E, Sham PC, Glover G, Murray RM (1995). Prenatal exposure to influenza epidemics and risk of mental retardation. *Eur Arch Psychiatry Clin Neurosci* **245:** 255–259.

The European Agency for the Evaluation of Medicinal Products (2003). Points to consider on the development of live attenuated influenza vaccines. EMEA/CPMP/BWP/2289/01.

Thraenhart O, Schley G, Kuwert E (1975). Isolation of influenza virus A/Hong Kong/1/68 (H3N2) from liquor cerebrospinalis of patients with CNS involvement. *Med Klin* **70:** 1910–1914.

Togashi T, Matsuzono Y, Narita M (2000). Epidemiology of influenza virus-associated encephalitis-encephalopathy in Hokkaido, the nothernmost island of Japan. *Pediatr Int* **42:** 192–196.

Voudris KA, Skaardoutsou A, Haronitis I, Vagiakou EA, Zeis PM (2001). Brain MRI findings in influenza A-associated acute necrotizing encephalopathy of childhood. *Eur J Paediatr Neurol* **5:** 199–202.

Ward A (1996). Neurovirulence of influenza A virus. *J NeuroVirol* **2:** 139–151.

Ward AC (1997). Virulence of influenza A virus for mouse lung. *Virus Genes* **14:** 187–194.

Yokota S, Imagawa T, Miyamae T, Ito S, Nakajima S, Nezu A, Mori M (2000). Hypothetical pathophysiology of acute encephalopathy and encephalitis related to influenza virus infection and hypothermia therapy. *Pediatr Int* **42:** 197–203.