

Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration

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One of the greatest influenza pandemic threats at this time is posed by the highly pathogenic H5N1 avian influenza viruses. To date, 61% of the 433 known human cases of H5N1 infection have proved fatal. Animals infected by H5N1 viruses have demonstrated acute neurological signs ranging from mild encephalitis to motor disturbances to coma. However, no studies have examined the longer-term neurologic consequences of H5N1 infection among surviving hosts. Using the C57BL/6J mouse, a mouse strain that can be infected by the A/Vietnam/1203/04 H5N1 virus without adaptation, we show that this virus travels from the peripheral nervous system into the CNS to higher levels of the neuroaxis. In regions infected by H5N1 virus, we observe activation of microglia and alpha-synuclein phosphorylation and aggregation that persists long after resolution of the infection. We also observe a significant loss of dopaminergic neurons in the substantia nigra pars compacta 60 days after infection. Our results suggest that a pandemic H5N1 pathogen, or other neurotropic influenza virus, could initiate CNS disorders of protein aggregation including Parkinson's and Alzheimer's diseases.

alpha-synuclein | Parkinson's disease | substantia nigra | stereology | encephalitis

At this time, one of the greatest worldwide pandemic threats is posed by the highly pathogenic H5N1 family of avian influenza A viruses; where to date, 61% of the 433 known human cases of H5N1 infection have proved fatal (1). Although most infections primarily affect the respiratory system, neurological symptoms have been associated with past influenza type A outbreaks, including the 1918 "Spanish" influenza pandemic (2, 3). In recent times, poultry (4) and humans (5, 6) infected by H5N1 have shown acute neurological signs ranging from mild encephalitis to motor disturbances to coma. However, despite these acute descriptions, no studies have examined the longer-term neurologic consequences of H5N1 infection in surviving hosts.

It has been hypothesized that viruses have an etiological role in the development of several neurodegenerative disorders including the two most common, Alzheimer's and Parkinson's diseases (7). Each of these disorders are characterized by neuronal loss, abnormal protein aggregations (proteinopathies), and activation of the microglia that act as the brain's innate immune system (8). Parkinson's disease (PD) is characterized by a loss of the pigmented cells located in the midbrain's substantia nigra pars compacta (SNpc), although cell loss has also been described in the locus coeruleus (9), the dorsal motor nucleus of the vagus nerve (10), and throughout the autonomic nervous system (11). In addition to neuronal loss, primary PD is also defined by the presence of proteinaceous inclusion bodies called Lewy bodies. Lewy bodies were first described and linked to PD by Frederic Lewy (12), and subsequently these have been shown to consist of a number of proteins including aggregated alpha-synuclein (13). Alzheimer's disease is characterized by a general neuronal loss, neurofibrillary tangles, and abnormal deposition

of proteins into cerebral plaques that are composed in part by beta-amyloid and alpha-synuclein. In this study, we directly tested whether the highly pathogenic A/VN/1204/04 H5N1 influenza virus could induce a neuronal pathology similar to that seen in proteinopathies.

Results

We first determined the infectivity of the highly pathogenic and neurotropic A/Vietnam/1203/04 (H5N1) virus by intranasally inoculating 225 6- to 8-week-old C57BL/6J mice (Jackson Laboratories) with the 50% mouse lethal dose (MLD₅₀) (14). Three days postinoculation (p.i.), approximately two-thirds of the mice demonstrated weight loss; by day 8 p.i., weight loss was >10% of initial body weight. All of the infected animals that showed weight loss showed neurological signs including ataxia, tremor, and bradykinesia. For approximately half of these animals, their symptoms became so severe that they had to be euthanized. The remainder showed milder neurological signs and recovered by day 21 p.i.

The neurological signs observed in the infected mice suggested involvement at several levels of the neuraxis. We mapped the progression of the H5N1 virus through the nervous system by using immunohistochemistry with an antibody to the influenza virus nucleoprotein (NP) (Table 1). H5N1 virus was first detected 2–3 days p.i. in the mesenteric and myenteric (Auerbach's) plexi of the enteric nervous system (Fig. 1A) and in neurons within the dorsal root ganglia (DRG) (Fig. 1B) of the peripheral nervous system. H5N1 virus was first observed in the CNS on day 3 p.i. in the brainstem solitary nucleus (Fig. 1C), which receives primary afferent signals from various visceral regions and organs, including lung and gut (15). On day 7 p.i., virus was found in the vagal and hypoglossal brainstem nuclei and in the midbrain locus coeruleus and SNpc (Fig. 1D). Outside of the brainstem and midbrain, H5N1 virus was detected in the periglomerular (Fig. 1E) and mitral (Fig. 1F) cells of the olfactory bulb and in neurons within the spinal cord (principally in the thoracic region) (Fig. 1G). On day 10 p.i., H5N1 infection had spread to all levels of the central neuraxis but did not involve all structures (Table 1, Fig. S1, and Fig. S2). By day 21, surviving animals

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Table 1. Semi-quantitative analysis of H5N1 expression in CNS and lung

Days p.i.	Lung	Ctx	OB	DRG	Thal	SN	LC	RN	Vest n	Pons	Solitary n	VII	X	XII
1	+	-	-	ND	-	-	-	-	-	-	-	-	-	-
2	+	-	-	ND	-	-	-	-	-	-	-	-	-	-
3	++	-	-	+	-	-	-	-	-	-	+	-	-	-
7	++	-	+	ND	-	+	+	-	+	-	++	-	++	++
10	+	++	++	++	++	++	++	+	++	++	+++	++	++	+++
21	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
90	-	-	-	ND	-	-	-	-	-	-	-	-	-	-

Semiquantitative immunohistochemical measurement of the percentage of anti-NP-positive cells at the indicated days p.i. with H5N1 virus. + = 0%–33%, ++ = 34%–67%, +++ = 67%–100%, – = none, ND = not determined. Ctx, cerebral cortex; OB, olfactory bulb; DRG, dorsal root ganglia; Thal, thalamus; SN, SNpc; LC, locus coeruleus; RN, red nucleus; Vest n, vestibular nucleus; solitary n, solitary nucleus; VII, facial nucleus; X, vagal nucleus; XII, hypoglossal nucleus.

displayed no visible neurological signs and H5N1 virus was not detected in the CNS. The pattern of infection in lung and brain suggested that active infection lasted ≈ 10 days, as described in humans (5). Additional images showing the pattern of infection are shown in Fig. S1.

In the CNS, the virus was detected in neurons (Fig. 1*H*) and microglia (Fig. 1*I*) but was not seen in astrocytes (Fig. 1*J*). The presence of virus in brainstem neurons was confirmed by transmission electron microscopy, which revealed structures of the shape and size characteristic of H5N1 influenza virus (16) in the cytoplasm (Fig. 1*K*), axoplasm (Fig. 1*L*), and nucleus.

Our observation of H5N1 virus first in the enteric and peripheral nervous systems and then slightly later in the CNS, first in the brainstem solitary nucleus and subsequently in other brainstem nuclei (Table 1, Fig. 1, and Fig. S1), suggests that the A/Vietnam/1203/04 virus may enter the CNS via cranial nerves, as other strains of H5N1 are reported to do (17, 18). This hypothesis was tested by using microfluidic chambers that allow neuronal cell bodies and their processes to be grown in one compartment while their processes extend through 150- μ m wide microgrooves into a separate process compartment (19). We added H5N1 virus to the process compartment for 1 h and detected virus in the cell bodies (Fig. 2*A*) and their processes (Fig. 2*B*) within the following 24 h. Because hydrostatic pressure differences between chambers would have confined extracellular virus to the process compartment, this finding showed that the virus detected in neuronal soma had been transported intracellularly. Direct exposure of cell bodies to H5N1 influenza also resulted in infection (Fig. 2*C*). These results demonstrate that H5N1 virus can infect neurons after peripheral infection and by hematogenous spread directly infecting cells as would occur after its release from dying cells.

One of the hallmarks of neurodegenerative disorders including Parkinson's and Alzheimer's disease is the aggregation of alpha-synuclein (20). This aggregation has been shown to depend on the phosphorylation of a number of serine residues in the synuclein protein (SYN), of which serine 129 appears to be most important (21). In 2–3-month-old uninfected C57BL/6J mice, we observed pSer129SYN in few, if any, neurons at any level of the central neuraxis, including the dopaminergic neurons of the substantia nigra pars compacta (Fig. 3*A*). However, in H5N1-infected mice, we observed pSer129SYN-positive neurons at various levels of the neuraxis including the brainstem and midbrain (Fig. 3*B*), hippocampus, and cortex. The phosphorylation of Ser129SYN appears to be directly related to H5N1 infection, because uninfected struc-

tures within the CNS did not show pSer129SYN up-regulation. Using NIH Image (version 1.63), we quantified the expression of p129SerSYN in olfactory bulb, hippocampus, locus coeruleus, brainstem and cerebellum. Animals infected with H5N1 infection had significantly increased expression of both cellular and secreted pSer129SYN at all levels of the neuraxis examined except for cerebellum (Table 2). In addition to pSer129SYN expression, we also noted aggregated alpha-synuclein in hippocampus (Fig. 3*C*), cortex, and brainstem, as seen in human proteinopathies such as Parkinson's and Alzheimer's diseases (20).

To determine whether H5N1 infection of the CNS resulted in cell death, we examined markers of apoptotic and necrotic cell death [caspase-3 activation (22) and Fluoro-Jade-B staining (23), respectively]. We also stereologically assessed dopaminergic neuron number in the substantia nigra pars compacta (SNpc). In regions of the brain where H5N1 virus had been detected, we found apoptotic (Fig. 3*D*) but not necrotic cells. We also noted activated microglia (Fig. 3*E*), which indicate an inflammatory process (24), in these regions. Microgliosis persisted in the CNS after the acute infection resolved and was observed during the entire 90-day study period (Fig. 3*F*). This prolonged inflammatory response is similar to that described in humans with idiopathic Parkinson's disease (25) and with parkinsonism induced by the neurotoxin MPTP (26). Cell counts of dopaminergic neurons in the SNpc 60 days after H5N1 infection demonstrated a 17% loss of dopaminergic neurons (8963 ± 384 DA neurons in control, 7481 ± 453 DA neurons in H5N1-infected mice, $n = 4$, $P \leq 0.016$).

Discussion

Reports of influenza-associated neurological syndromes are found as far back as 1385 and have continued through more recent influenza outbreaks (27). There is a substantial amount of evidence that influenza can directly lead to encephalitis (5, 28–33), although the link with development of neurodegenerative diseases including Parkinson's disease are controversial. Much of the linkage of parkinsonism with influenza infection are based on the postencephalic parkinsonism that followed an outbreak of von Economo's encephalopathy (EL) subsequent to the 1918 influenza pandemic (34). This includes epidemiological data (2, 3) and physical findings of type A influenza antigens in EL patients (35). Evidence against the role of influenza as a parkinsonian agent include the lack of viral RNA recovered from brains of postencephalic parkinsonian patients (36), the absence of any known mutations that

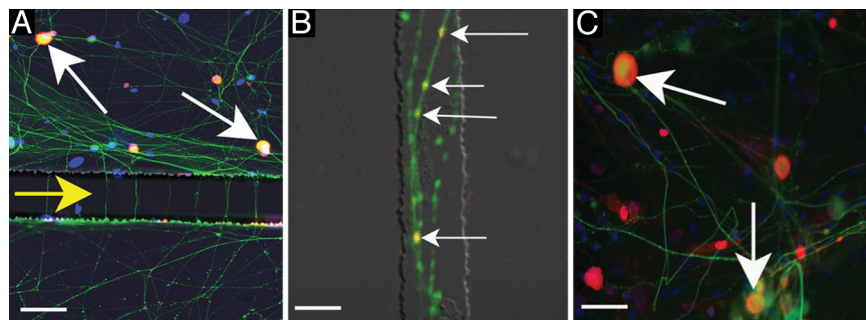


Fig. 2. Transport of H5N1 virus through axons in microfluidic chambers. (A) A/Vietnam/1203/04 (H5N1) influenza virus (2×10^6 PFU suspended in 20 μ L serum-free medium) was added to the process compartments of microfluidic chambers containing freshly dissociated DRG (55). After 1 h, the process compartment was washed twice by adding 130 μ L serum-free medium to the top reservoir and collecting the flow-through in the bottom reservoir. One hundred thirty microliters medium containing serum was then added to the top and bottom reservoirs of the process compartments to maintain hydrostatic balance. Twenty-four hours after addition of H5N1 virus to the process compartment (bottom), H5N1 NP is detected in neuronal bodies within the somal compartment (white arrows). Yellow arrow shows axons (green, beta3-tubulin) traversing the series of 150 μ m-wide grooves between the compartments. (B) High power photomicrograph of DRG axons (green, beta3-tubulin) growing through the 150- μ m grooves. Virus particles (arrows) within the axons appear yellow because of colocalization with neuronal beta3-tubulin. (C) Neuronal bodies (green) are infected after direct exposure to H5N1 virus (red, viral NP protein; arrows). Yellow indicates colocalization of proteins. [Scale bars: 160 μ m (A); 6 μ m (B); 60 μ m (C).]

cells, respectively were examined as described in ref. 22. Alpha synuclein (BD Biosciences, cat# 610786) and pSer129 SYN (WAKO, monoclonal antibody #014–20281) immunocytochemistry were performed on frozen sections using standard protocols (53). Transmission electron microscopy was performed as described in ref. 54.

DRG Neuron Culture and Microfluidic Chamber Studies. Dissociated DRG neurons were prepared as described in ref. 55. Dissociated cells (60,000) were plated into the “cell body compartment” of microfluidic chambers (19). After 30 min to allow cell attachment, cultures were rinsed and 150 μ L fresh media was added. At this time, 150 μ L fresh medium containing 10 ng/mL nerve growth factor (NGF 2.5s, N6009, Sigma) was added to the “process compartment” of the microfluidic chamber. Cultures were incubated for 12 days at 5% CO₂, after which chambers were infected with 2×10^6 PFU of H5N1 in 20 μ L media. After 1 h, the microfluidic axonal chamber was washed 2 $\times$ by adding 130 μ L serum free media to the top reservoir and collecting the flow through in the bottom reservoir. 130 μ L media with serum was then added to the top and bottom reservoirs on the axonal side to maintain the hydrostatic balance. At the designated times, cells were rinsed with 1 $\times$ PBS and fixed by 4% paraformaldehyde for 1 h. Virus was detected by immunohistochemical methods as described above.

Quantification of the Phosphorylated α -Synuclein Expression. To ensure consistency in this analysis, all sections were processed on the same day using the same solutions and antibody preparations, including lot numbers. Sixteen-micrometer coronal frozen sections were washed with 0.1 M PBS. One set of section were incubated with 10 μ g/mL proteinase K (Invitrogen)/0.1 M PBS solution for 10 min at room temperature, and another set of adjacent sections were washed with 0.1 M PBS without proteinase K. These different methods specifically allow identification of insoluble versus soluble pSer129SYN, respectively (56). All sections were blocked with M.O.M blocking solution (Vector Laboratories) for 1 h and incubated overnight at 4 $^{\circ}$ C with anti-phospho Ser129 antibody (1:1000, clone pSYN#64, WAKO) diluted in M.O.M diluent solution. Sections were incubated with a biotinylated secondary antibody for 30 min and then incubated with avidin-biotin complex (ABC kit, Vector Laboratories) for 15 min and washed in 0.1 M PBST 5 $\times$ for 5 min each and developed with 3–3' diaminobenzidine (Sigma) as the chromogen. Sections were dehydrated, cleared and mounted with Permount (Sigma). Images were captured at 10 $\times$ using the virtual slice program in the Microbrightfield system (Microbrightfield) attached to an Olympus BX60 microscope. All images for analysis were taken at the same light intensity at one sitting to control for variations in bulb intensity and camera chip differences. All images were saved as a .tif file and optical densities of the rostral hippocampus, olfactory bulb, locus coeruleus, solitary nucleus, and cerebellum were obtained on raw

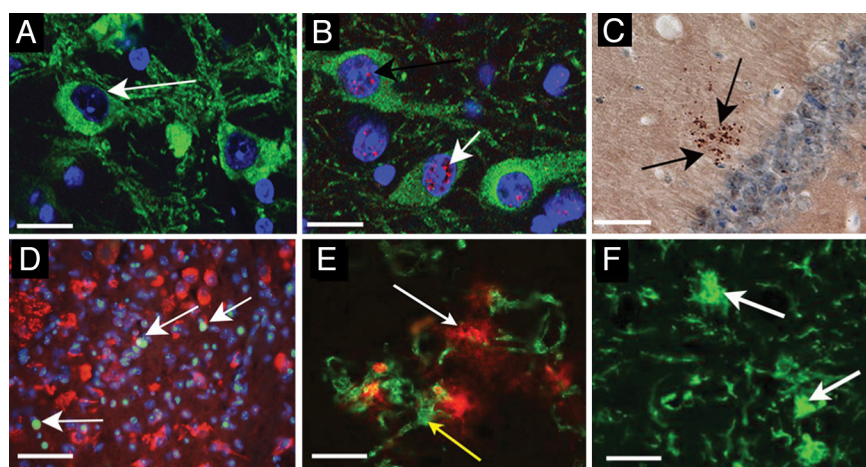


Fig. 3. A/Vietnam/1203/04 (H5N1) influenza virus induces a parkinsonian phenotype. (A) In uninfected C57BL/6J mice, no pSer129SYN is seen in the dopaminergic neurons of the SNpc (green). (B) At day 60 p.i., pSer129SYN (white arrows) is seen in the nuclei of dopaminergic neurons (green) in the SNpc. (blue, DAPI nuclear stain.) (C) At day 90 p.i., aggregated alpha synuclein (arrows) is seen in swollen neurites in the hippocampus. (D) At day 10 p.i., activated caspase-3 (arrows) is seen in brainstem regions that contain NP protein (red). The cells expressing activated caspase-3 do not appear to be NP-positive. (E) At day 10 p.i., activated microglia marked by anti-Iba-1 (green; yellow arrow) surround infected cells (red; white arrow). (F) At day 60 p.i., activated microglia (arrows) persist in midbrain regions (and all other regions) infected by H5N1. Findings were similar at day 90 p.i. [Scale bars: 30 μ m (A and B); 50 μ m (C); 40 μ m (D); 35 μ m (E); 50 μ m (F).]

Table 2. Relative density measurements of pSer129SYN in brain

	no PK			pK-treated		
Structure	Control	H5N1	Statistics	Control	H5N1	Statistics
olfactory bulb	100 ± 19	178 ± 27	$P \leq 0.05$	100 ± 19	189.21 ± 23	$P \leq 0.01$
hippocampus	100 ± 21	277 ± 21	$P \leq 0.001$	100 ± 21	244 ± 25	$P \leq 0.001$
locus coeruleus	100 ± 20	120 ± 12	n.s.	100 ± 7	168 ± 10	$P \leq 0.001$
solitary nucleus	100 ± 18	190 ± 13	$P \leq 0.001$	100 ± 7	163 ± 8	$P \leq 0.001$
lower brainstem	100 ± 17	274 ± 37	$P \leq 0.001$	100 ± 21	135 ± 14	n.s.
cerebellum	100 ± 8	100 ± 3	n.s.	100 ± 16	108 ± 12	n.s.

Relative density measurements of p129SerSYN in brain. Both soluble p129SerSYN (noPK) and insoluble (pK) forms of this phosphoprotein were determined. Statistical analysis was performed using GraphPad software.

images using the NIH image program. The density of the background from each section was also obtained and subtracted from the region density to give a final number. Statistical analysis was performed using GraphPad statistical software.

Stereological Assessment of SNpc DA Neuron Number. SNpc dopaminergic neurons were stereologically assessed using the optical fractionator as described in ref. 57.

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