Lethal avian influenza A (H5N1) virus induces ataxic breathing in mice with apoptosis of pre-Botzinger complex neurons expressing neurokinin-1 receptor

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Lethal avian influenza A (H5N1) virus induces ataxic breathing in mice with apoptosis of pre-Botzinger complex neurons expressing neurokinin-1 receptor. Am J Physiol Lung Cell Mol Physiol 313: L772–L780, 2017. First published July 20, 2017; doi:10.1152/ajplung.00145.2017.—Lethal influenza A (H5N1) induces respiratory failure in humans. Although it also causes death at 7 days postinfection (dpi) in mice, the development of the respiratory failure and the viral impact on pre-Botzinger complex (PBC) neurons expressing neurokinin 1 receptor (NK1R), which is the respiratory rhythm generator, have not been explored. Body temperature, weight, ventilation, and arterial blood pH and gases were measured at 0, 2, 4, and 6 dpi in control, lethal HK483, and nonlethal HK486 viral-infected mice. Immunelectron microscopie (IR) of PBC NK1R, H5N1 viral nucleoprotein (NP), and active caspase-3 (CASP3; a marker for apoptosis) were detected at 6 dpi. HK483, but not HK486, mice showed the following abnormalities: 1) gradual body weight loss and hypothermia; 2) tachypnea at 2–4 dpi and ataxic breathing with long-lasting apneas and hypercapnic hypoxemia at 6 dpi; and 3) viral replication in PBC NK1R neurons with NK1R-IR reduced by 75% and CASP3-IR colabeled at 6 dpi. Lethal H5N1 viral infection causes tachypnea at the early stage and ataxic breathing and apneas (hypercapnic hypoxemia) leading to death at the late stage. Its replication in the PBC induces apoptosis of local NK1R neurons, contributing to ataxic breathing and respiratory failure.

Materials and Methods

The present study was approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the Lovelace Respiratory Research Institute. All facilities were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. BALB/c female mice experiments were conducted in the Animal Biosafety Level 3 enhanced (ABSL-3+) facility. Guidelines for mice housing, environment, and comfort described in the Guide for the Care and Use of Laboratory Animals (7th Ed., National Institutes of Health) were strictly followed. Animals. A total of 106 pathogen-free female BALB/c mice (~4 wk old) were purchased from Charles River Laboratories (Wilmington, MA) and quarantined before the experiments. Mice had access to food and water ad libitum with temperature and humidity ranging from 16 to 22°C and 30 to 65%. Mice were identified by an IPTT-300 implantable programmable temperature and identification transponder (Bio Medic Data Systems, Seaford, DE). They were individually placed in a whole body, unrestrained, plethysmograph chamber (volume: 450 ml; Model PLY3211; Buxco Electronics, Troy, NY) for ~45 min twice a day for 2 continuous days before the experiment was performed. The chamber was continuously flushed with normoxic (21% O2–79% N2) gas mixtures at 500 ml/min.

Experimental protocols. Series I was designed to compare the impacts of H5N1 viral infection on body weight, body temperature, and ventilation at 0, 2, 4, and 6 dpi (n = 7, 7, 8, and 10 each time point) among Ctrl, HK486, and HK483 mice. At the experimental day, ventilation was recorded in the mice placed in the chamber after the body weight and temperature were measured. After completion of the experiment, three mice from the HK486 and HK483 groups at each time point, respectively, were euthanized with the lungs fixed with 10% buffered formalin. Histological examination of the development of pneumonia following the viral infection was conducted over the 6 days.

Series II was conducted to compare the effects of HK483 and HK486 viral infection on 1) arterial blood pH and gases; 2) pulmonary and brain edema; and 3) lung viral titer. Lethal H5N1 viral infection at 6 dpi has shown hypoxemia, pulmonary infiltration, and brain

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**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

![Graph C](image3)

**D**

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perivascular edema (1, 9, 12, 35, 47, 48) in humans, which are closely related to respiratory modulation. After measuring ventilation at each time point in study series I, the mice were anesthetized with urethane to collect arterial blood from the femoral artery and euthanized to harvest the right lung for analyzing the viral titer and the left lungs and the brain for estimating the wet-to-dry wt ratios.

Series III aimed to test whether HK483, but not HK486, virus would replicate in PBC neurons expressing NK1R to induce apoptosis and affect NK1R expression at 6 dpi when respiratory failure occurred. The brainstems were collected from five HK483 and five HK486 mice and paraffin embedded. Coronal sections containing PBC were cut (10-μm thick) and underwent immunohistochemical processes to detect the following immunoreactivities (IRs) in the PBC: 1) H5N1 viral nucleoprotein (NP) to detect H5N1 viral infection; 2) neural NK1R to label respiratory rhythm generators; 3) active caspase-3 (CASP3; a marker of apoptosis) to mark apoptosis (17, 20, 79); and 4) the combination of J with 2 or 3.

Viruses and cells. Avian influenza A H5N1 viruses were obtained from the Centers for Disease Control and Prevention [Centers for Disease Control and Prevention (CDC), Atlanta, GA]. They were propagated from the CDC stock in eggs twice to produce working stocks, aliquoted, titrated by plaque assay on Madin-Darby canine kidney (MDCK) cells, and stored at −80°C (70).

Viral infection of mice and behavioral observation. After anesthesia with isoflurane, 50 μl of vehicle (HK483 or HK486 virus) were given intranasally (100 plaque-forming unit) via dropwise application to the nares as previously reported (50) and the mice were divided into three groups: control (Ctrl), HK486, and HK483. Behavioral observations were conducted twice daily from 0 up to 6 dpi. They included body temperature and recording of the onset, severity, and duration of all visible changes such as abnormal respiration (cough and sneezing), excretions, behavioral characteristics, and neurological signs (i.e., paresis, torticollis, seizures, and paralysis).

Measurements of Ve and respiratory rhythm variability. Following stabilization, minute ventilation (Ve), respiratory frequency (fR), tidal volume (VT), respiratory intervals were recorded for 30 min by using the plethysmography. The variation of respiratory intervals is a more sensitive index than fR in reflecting a change of respiratory control (34). We applied the Poincare analysis using 2500 consecutive respiratory intervals from breaths (21% O2-79% N2) in each animal in which the duration of each breath was plotted vs. the next as previously reported (6). The width of the standard deviation was calculated perpendicularly to (SD2) and along the line of identity (SD2). The temperature inside the chamber was maintained at ∼30°C as reported before (5, 28). All studies were performed during 0900 and 1700 to avoid any influence from the circadian rhythm.

Blood sample collections and measurements of pH and blood gases. The mice were appropriately anesthetized with urethane (1,200 mg/kg ip) with the right femoral artery isolated and cannulated. Arterial blood was sampled (100 μl) to detect pH and blood gases using a blood gas analyzer (GEM Premier 3000; Instrumentation Laboratory, Lexington, MA).

Virus titer in the lungs. Plaque assay was performed in MDCK cells to quantify the multiplicity of viral infection in the right lungs. In brief, after euthanasia, the right lungs from each mouse were harvested and homogenized in 1.0 ml of PBS with one 5-mm stainless steel bead and homogenized with a Qiagen TissueLyser (Qiagen, Valencia, CA) for 2 min at 30 Hz/s. Homogenized material was spun down, and 100 μl supernatant with 10-fold series dilution were applied to preseeded 12-well plates of MDCK cells (95% confluent) and then overlaid with agar containing 3 μg/ml of trypsin (Sigma-Aldrich, St. Louis, MO). Three days later, the plaque-forming unit was counted after fixation, removal of the agar, and staining with 1.6% wt/vol crystal violet.

Histopathological characteristics. The right lung lobe of each mouse was fixed in 10% buffered formalin, embedded in paraffin, divided into tissue sections (3-mm thick), stained with hematoxylin and eosin, and subjected to routine histological examination.

Lung and brain water contents. The left lungs and brain were harvested and weighed with an electronic balance. The wet sample was dried in an isothermoven (Model No. 97-920-1; Fisher Scientific, Pittsburgh, PA) at 60°C for 48 or 72 h. The tissues were weighed once every day after drying in the oven until the final two weights of the tissues became the same, and this weight was defined.
as dry weight. The wet wt-to-dry wt ratio was calculated to assess the pulmonary and brain edema.

**Immunofluorescence and digital image acquisition.** Two coronal PBC sections at 1.7 mm rostral to obex (27) from each HK483 and HK486 mouse were dewaxed and rehydrated. Antigen recovery was performed by using preheated sodium citrate buffer (10 mM pH 6.0) for 10 min in the microwave. Sections were permeabilized, and nonspecific antibody binding sites were blocked in blocking buffer (3% BSA, 2% normal goat serum, and 0.3% Triton X-100 in PBS) at room temperature for 1 h. Sections were then incubated with primary antibody mixtures at 4°C overnight, washed, and then incubated with secondary antibodies conjugated with Alexa Fluro 488 or 594 at room temperature for 1 h. Coverslips were mounted on stained sections with anti-fade reagent (Invitrogen). The following antibodies were utilized: 1) the nucleoprotein of H5N1 influenza A (mouse-anti-NP; MAB8251; EMD Millipore); 2) neurokinin-1 receptor (guinea pig anti-NK1R; AB15810; EMD Millipore); and 3) active caspase-3 (rabbit anti-CASP3, AB2303; Abcam). Digital micrographs of the PBC region were acquired using a ×10 objective with a digital camera (AxioCam HRm; Zeiss) connected to an epifluorescence microscope (Axioplan 2 FS; Zeiss).

**Data acquisition and statistical analysis.** Raw data of the airflow were digitized, monitored, and recorded by PowerLab/8sp (ADInstruments, Colorado Springs, CO). Respiratory variables including VT, fR, and TE were averaged over a 10-min period, and variables reflecting the variation of the respiratory intervals (SD1 and SD2) were derived from 2,500 consecutive respiratory intervals during which the animal breathing was relatively stable.

![Fig. 3. Influence of H5N1 viral infection on variation of the respiratory intervals. A: typical Poincare plots of the variation of the respiratory intervals in an HK486 and an HK483 mouse at 0, 2, 4, and 6 dpi. The area of the ellipse describes the distribution of the points with the width of the standard deviation perpendicular to (SD1 = 1) and along the line of identity (SD2 = 2). B: Group data of the variability of baseline respiratory intervals (2,500 intervals). Data are means ± SE; n = 7, 7, 8, and 10 for Ctrl, HK486, and HK483 mice, respectively. *P < 0.01, compared with 0 dpi; †P < 0.01, compared with Ctrl and HK486 on the given day; #P < 0.05 vs. the previous day in HK483 mice.](http://ajplung.physiology.org/)

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An apnea was defined as $T_e$ duration approximately threefold of a normal $T_e$ duration (28, 44), and its numbers in each animal were counted during the 30-min recording period. The optical density of PBC NK$_R$-IR alone and coupled with CASP3-IR or NP-IR was analyzed. NK$_R$-IR was measured and averaged from the micrographs of the PBC as previously described (49). The colocalizations of NK$_R$ + NP-IR and NK$_R$ + CASP3-IR were expressed as percentages (colabeling area over the area with positive NK$_R$-IR). All group data are reported as means ± SE or presented in box and whisker plots in some figures. Nonparametric (Wilcoxon method) was used to compare the number of apneas, $T_e$ duration at 6 dpi, and the immunoreactivity data. Two-way ANOVA with repeated measures was used to analyze the significant differences of other variables at 0, 2, 4, and 6 dpi among the three groups. Tukey’s test was utilized for specific comparisons between individual groups. $P < 0.05$ were considered significant.

RESULTS

HK483 viral infection induced abnormal behaviors. At 2 dpi, the mice infected by HK483 or HK486 virus showed no discernible behavior abnormalities as compared with Ctrl mice. However, HK483 mice presented less movement, loss of appetite, torticollis, and neurological signs, such as tremor at 6 dpi. As exhibited in Fig. 1, A and B, both body temperature and body weight were not significantly different between Ctrl and HK486 mice over the infection period. However, a decrease in body weight and body temperature started at 4 dpi and became worse at 6 dpi in HK483 mice. No coughing and/or sneezing were observed in all of the mice tested. Viral titers in the lungs were similar between HK483 and HK486 mice at 4 and 6 dpi although they were relatively higher in the former than the latter at 2 dpi (Fig. 1C). As shown in Fig. 1D, elevation of inflammatory cells occurred at 2 dpi. Various degrees of bronchitis and epithelial necrosis were observed with remarkable tissue damage in the lower respiratory tract within 4 dpi that became worse at 6 dpi. The changes were characterized by interstitial inflammation, hyaline layer formation, varying degrees of alveolar edema, hemorrhage, and inflammation. Consistent with previous results reported in mice (13, 18, 50), the pulmonary inflammation induced by HK486 and HK483 was, in general, not strikingly different between HK486 and HK483 mice.

Apneas (ataxic breathing) was responsible for the ventilatory failure in HK483 mice. As presented in Fig. 2, over the infection period, $V_e$, $f_R$, and $V_T$ were not different between Ctrl and HK486 mice. However these values, especially $f_R$, in the mice infected with HK483 virus were initially increased at 2 and/or 4 dpi and then returned to the levels before infection at 6 dpi. Interestingly, only HK483 mice presented significantly diminished variation of respiratory rhythm including $SD_1$ and $SD_2$ at 2 and 4 dpi, but the variability became substantially enhanced later, especially $SD_1$ at 6 dpi (Fig. 3, A and B), indicating a great irregularity of respiratory cycle. In agreement, HK483 but not HK486 mice showed high irregularity of $f_R$ (Fig. 4A) at 6 dpi that was associated with greatly increased apneic numbers and prolonged apneic duration (Fig. 4B). The longest apneic durations were 4.2 $\pm$ 1.1 s in HK483 mice, while these values were 1.4 $\pm$ 0.4 and 1.2 $\pm$ 0.5 s in Ctrl and HK486 mice ($P < 0.01$).

HK483 viral infection caused hypercapnic hypoxemia. We compared arterial blood gases/pH among the three groups (Fig. 5) and found that blood gases/pH changes were similar between Ctrl and HK486 mice over the infection period. In sharp contrast, HK483 virus induced a significant decrease in $pH_a$, $PaO_2$, and $SaO_2$, with a remarkable increase in $PaCO_2$ at 4 and/or 6 dpi. Owing to the inhibitory impact of the anesthetic on respiration, our $PaO_2$ and $SaO_2$ data obtained in the anesthetized

A

![Flow ml/s](Fig_4A.png)

B

![Apnea number #/10 min](Fig_4B.png)
animals were relatively lower as compared with the conscious state. Nevertheless, the difference of PaO2 and SaO2 between HK486 (Ctrl) and HK483 reflects the impacts of lethal H5N1 viral infection on these variables.

HK483 viral infection induced pulmonary edema compared with HK486 virus. We compared wet wt-to-dry wt ratios of the lungs and the brain at the four time points in the two infected groups. As listed in Table 1, compared with 0 dpi, HK486 failed to induce a significant change in both wet wt-to-dry wt ratios of the lungs and the brain at 2, 4, and 6 dpi, while HK483 viral infection significantly increased the wet wt-to-dry wt ratio of the lungs at 6 dpi.

HK483 virus replicated the PBC to cause apoptosis and reduce NK1R neural population. Our immunohistochemical data illustrated in Fig. 6 could be summarized by the statements that follow. First, NP-IR was observed in the PBC, particularly local NK1R neurons, only in HK483 mice. Second, PBC NK1R-IR expressed predominantly in a “cell-shape pattern” in HK486 mice, but in a “fragmentation-like pattern” in HK483 mice. Importantly, the optical density of PBC NK1R-IR was reduced to 25% by HK483 virus compared with HK486 virus, ~61% of which showed colabeling with NP-IR. Third, CASP3-IR was detected in the PBC, including NK1R neurons, and 26% of the remaining 25% NK1R-IR positive area was colabeled with CASP3-IR.

DISCUSSION

Previous studies have indicated that respiratory failure is the major cause of death in patients with lethal H5N1 viral infection (1, 3, 9, 35, 47, 48). The lethality of the virus isolated from human cases in H5N1 outbreaks has also been reported in animals (13, 18, 21, 31), such as the death at 7 dpi in mice (50). However, the development of the respiratory failure to a life-threatening degree over the infection period has not been unfolded. One of the novel findings in this study is that respiratory rhythm disorder is the major pathological consequence induced by lethal HK483 viral infection. This disorder is characterized by a shift from tachypnea (2 dpi) to a frequent appearance of long-lasting apneas with ataxic breathing (6 dpi) before the death. Normal variability of breath intervals represents an optimal breathing control ranging from an absence of any variability to a clearly periodic breathing pattern as the result of excitatory and inhibitory inputs from many neural feedback loops at different states (51). Thus the shift from tachypnea to ataxic breathing noted in the present study points to a neural origin of the respiratory disorders induced by HK483 viral infection. This respiratory shift is further confirmed by our arterial blood pH/gases data. We found that HK483 viral infection at 2 dpi induced tachypnea without a change in blood gases/pH, indicating an ability of respiratory compensatory response at this moment to keep blood gases homeostatic. However, this compensatory ability declined thereafter to lead to respiratory failure at 6 dpi, at which point ataxic breathing and long-lasting apneas appeared associated with hypercapnic hypoxemia and acidosis. It is well documented that hypoxia and hypercapnia synergistically stimulate ventilation (7, 8, 11, 37, 43). The failure of sufficient ventila-

Table 1. Wet wt-to-dry wt ratios of lung and brain tissues

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<th>dpi</th>
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<td>6</td>
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Values are means ± SE. *P < 0.05, 6 days postinfection (dpi) vs. 0 dpi.
tory response to the hypoxic hypercapnia and the appearance of ataxic breathing are the basic features of the respiratory failure in HK483 mice. Collectively, our results reveal, for the first time, a shift from the initial tachypnea at 2 dpi to the ataxic breathing (apneas, especially long-lasting apneas) associated with hypercapnic hypoxemia before death.

The mechanisms by which lethal H5N1 virus induces tachypnea at the early stage of the infection have not been determined, but they are probably due to the infection of bronchopulmonary C fibers. It is well known that bronchopulmonary C fibers can trigger tachypnea (10, 23, 42) when they are moderately stimulated. Actually, replication of lethal HK483 virus has been observed in vagal sensory C fibers in mice at 2 dpi (50). Moreover, an overexpression of substance P in bronchopulmonary C fibers after HK483 viral infection supports a stimulatory effect of lethal H5N1 virus on these fibers (50). Evidence is accumulating to show that the overexpression of substance P in bronchopulmonary C fibers is the result of sensitization and excitation of these fibers after exposure to cigarette smoke, prenatal nicotinic exposure, and respiratory syncytial virus infection in rats and mice (29, 45, 46, 52). These lines of information are favorable for the contribution of the infected bronchopulmonary C fibers to the tachypnea, although further studies are warranted to provide the direct evidence.

PBC NK₁R neuron has been thought to play a key role in respiratory rhythm generation (32). Selective lesion of these neurons induced ataxic breathing with apneas associated with hypercapnic hypoxemia before death.

![Fig. 6. Pre-Botzinger complex (PBC) immunoreactivity (IR) of neurons expressing neurokinin 1 receptor (NK₁R), nucleoprotein (NP), caspase-3 (CASP3) alone, and NK₁R colocalization with NP or CASP3 in HK483 and HK486 mice at 6 dpi.](image)

**A**

- Typical micrographs from the PBC. NK₁R-IR, but not NP-IR (1st column) and CASP3-IR (2nd column), is detectable in the PBC of a HK486 mouse. In sharp contrast, a remarkable expression of NP-IR (3rd column) and CASP3-IR (4th column) alone and coupled with NK₁R is observed in a HK483 mouse. *Inset*: higher power images more clearly shows the apoptotic NK₁R neurons.

**B**

- Group data of PBC NK₁R-IR optical density, ratio of colocalized positive NP-IR in NK₁R areas, and ratio of colocalized positive CASP3-IR in NK₁R areas, respectively; n = 5 and 5 for HK486 and HK483 mice. OD, optical density. *P* < 0.01 for HK483 vs. HK486. Scale bar = 50 μm. Note: the values from HK486 mice in the middle and right are 0.

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HK483, but not HK486 virus, 1) replicated in the PBC, especially local NK,R neurons; 2) induced apoptosis of these neurons; and 3) reduced NK,R expression by 75%. In addition, PBC NK,R-IR expression in HK486 mice presents a “cell-shape pattern” that becomes a “fragmentation pattern” in HK483 mice. Neural apoptosis may be accountable for the HK483-induced shift from the “cell-shape pattern” to the “fragmentation pattern.” As previously reported (15, 41), apoptosis greatly affects localization of cell membrane proteins, including membrane receptors, as a result of loss of plasma membrane asymmetry and bleb of plasma membrane. Because of the key role of PBC NK1R neurons in generating respiratory rhythm and maintaining normal breathing, the abnormal NK1R expression supports the assumption that the respiratory failure by HK483 results, at least in part, from viral replication and subsequent apoptosis in PBC NK1R. Further studies are necessary to determine whether protection of PBC NK,R neurons against the viral infection-induced apoptosis prevents the lethality of H5N1 viral infection. The route by which the lethal H5N1 virus enters the central nervous system has been debatable and cannot be determined in this study. Some investigators believe that viral entry into the brain is mainly via the olfactory system (25, 26, 33), while others suggest other peripheral nerve fibers by which the virus enters the CNS, including trigeminal and sympathetic nerves (26), vagal efferents (18), and vagal sensory C fibers (50).

An increased wet wt-to-dry wt ratio of the lungs, but not the brain, was observed at 6 dpi with HK483 viral infection in this study, indicating a pulmonary edema, coinciding with a higher number of inflammatory cells in bronchoalveolar lavage fluid in HK483 than HK486 mice at 6 dpi (unpublished observation by J. Zhuang, N. Zang, and F. Xu). The pulmonary edema likely aggravates the severity of hypoxemia at 6 dpi in HK483 mice. Lethal H5N1 viral infection could also produce a body weight loss of 25–30% at 5–7 dpi in mice, cats, ferrets, and macaques (2, 22, 36, 39). In agreement, we found a loss of body weight (10–25%) at 4–6 dpi in this study. Fever was also observed in ferrets, cats, and macaques (19, 22, 24, 36, 39); however, a body temperature drop in mice was observed in this study as previously reported (50). The discrepancy seems to be species dependent. Taken together, the presence of decreased body temperature and pulmonary edema at the late stage of the infection may also contribute to the respiratory failure induced by lethal H5N1 viral infection.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.Z. and F.X. conceived and designed research; J.Z., N.Z., and C.Y. performed experiments; J.Z., N.Z., and C.Y. analyzed data; J.Z., N.Z., and F.X. interpreted results of experiments; J.Z. prepared figures; J.Z., N.Z., and F.X. drafted manuscript; J.Z. and F.X. edited and revised manuscript; F.X. approved final version of manuscript.

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