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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Running Head: Viral Infection and Breathing in Mice

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Abstract

Background.
Lethal influenza A (H5N1) induces respiratory failure in humans. Although it also causes death at 7 day 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.

Methods.
Body temperature, weight, ventilation, arterial blood pH and gases were measured at 0, 2, 4, and 6 dpi in control, lethal HK483 and non-lethal HK486 viral infected mice. Immunoreactivities (IR) of PBC NK1R, H5N1 viral nucleoprotein (NP), and active caspase-3 (CASP3, a marker for apoptosis) were detected at 6 dpi.

Results.
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 co-labeled at 6 dpi.

Conclusion.
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.

Key Words
Lethal viral infection; respiratory rhythm; respiratory failure; hypoxemia; apoptosis
Introduction

Patients infected with lethal avian influenza H5N1 viruses present shortness of breath (tachypnea) and cough during the first 6 days postinfection (dpi) and subsequently develop respiratory failure (hypoxemia), leading to death (60% mortality) several days later (1, 9, 35, 47, 48). Influenza viruses A/Hong Kong/483/97 (HK483, lethal H5N1) and A/Hong Kong/486/97 (HK486, nonlethal H5N1) isolated from human cases are known to possess dissimilar outcomes in experimental murine infection, despite nearly identical genetic compositions. For example, HK483 and HK486 virally infected mice show similar viral titer in lungs and pulmonary inflammation; however, only the former causes death often at 7-8 dpi in mice (13, 18, 50), ferrets (31), and cats (21). The development of these respiratory disorders to a life-threatening degree, which is a critical issue for revealing the pathogenesis of the respiratory failure, remains to date unexplored.

Lethal H5N1 virus is neurotropic. H5N1 infected cells are detected in the brains of acutely infected birds, mice, ferrets, and humans (13, 18, 30, 31) and replication of lethal A/Vietnam/1203/04 H5N1 virus is undetectable in brainstem neurons until 4 dpi in mice (18). More importantly, apoptosis in brain neurons has been observed at 4 dpi in H5N1 infected mice (4, 13). The pre-Botzinger complex (PBC) (32), particularly its neurons expressing neurokinin 1 receptor (NK1R), are essential for respiratory rhythm-generation. Lesion of these neurons induces ataxic breathing with apneas associated with hypercapnic hypoxemia (14). The ataxic breathing and apneas solely observed at 6 dpi of HK483 virus in our pilot study raised a
fundamental question as to whether the respiratory rhythmic disorder was associated with viral replication and apoptosis occurrence in PBC NK$_1$R neurons.

Materials and Methods

Animals

The present study was approved by the Institutional Animal Care and Use Committee (IACUC) 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 (AAALAC) 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 Edition, National Research Council) were strictly followed.

A total of 106 pathogen-free female BALB/c mice (~4 weeks old) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and quarantined before the experiments. Mice had access to food and water ad libitum with temperature and humidity ranging from 16–22°C and 30–65%. Mice were identified by an IPTT-300 implantable programmable temperature and identification transponder (Bio Medic Data Systems, Inc, Seaford, Delaware). They were individually placed in a whole-body, unrestrained, plethysmograph chamber (volume 450 ml, model PLY3211, Buxco Electronics Inc., Troy, NY) for ~45 min twice a day for two continuous days before performing the experiment. The chamber was continuously flushed with normoxic (21% O$_2$ and 79% N$_2$) gas mixtures at 500 ml min$^{-1}$. 
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 measuring the body weight and temperature. 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 six 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 perivascular edema (1, 9, 12, 35, 47, 48) in humans that 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:dry 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, 40); and 4) the combination of 1) with 2) or 3).

Viruses and cells

Avian influenza A H5N1 viruses were obtained from the 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 (50).

Viral infection of mice and behavioral observation

After anesthesia with isoflurane, 50 μl of vehicle (HK483 or HK486 virus) was intranasally given (100 PFU) via dropwise application to the nares as previously reported (50) and the mice were divided into three groups: 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 $V_E$ and respiratory rhythm variability

Following stabilization, minute ventilation ($V_E$), respiratory frequency ($f_R$), tidal volume ($V_T$), expiratory duration ($T_E$), and respiratory intervals were recorded for 30 minutes by using the plethysmography. The variation of respiratory intervals is a more sensitive index than $f_R$ in
reflecting a change of respiratory control (34). We applied the Poincare analysis using 2500 consecutive respiratory intervals from breaths (21% O₃ + 79% N₂) in each animal in which the duration of each breath was plotted versus the next as previously reported (6). The width of the standard deviation was calculated perpendicularly to (SD1) and along the line of identity (SD2). The temperature inside the chamber was maintained at ~30.0°C as reported before (5, 28). All studies were performed during 9:00 and 17:00 hours 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 (1200 mg kg⁻¹, 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 Lab., 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 Inc., Valencia, CA) for 2 minutes at 30 Hz/sec. Homogenized material was spun down and 100 µl supernatant with ten-fold series dilution was 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 (PFU) was counted after fixation, removal of the agar and staining with 1.6% w/v 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 (H&E), 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 isotemperature oven (Fisher Scientific Inc., Model NO. 97-920-1, Pittsburgh, PA, USA) 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:dry 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 pre-heated sodium citrate buffer (10 mM, pH 6.0) for 10 min in the microwave. Sections were permeabilized and non-specific antibody binding sites blocked in blocking buffer (3% BSA, 2% normal goat serum, 0.3% Triton X-100 in PBS) at room temperature for 1 hour. 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 hour. Cover slips were mounted on stained sections with anti-fade reagent (Invitrogen, USA). 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 10X objective with a digital camera (AxioCam HRm, Zeiss, Germany) connected to an epifluorescence microscope (Axioplan 2 FS, Zeiss, Germany).

**Data acquisition and statistical analysis**

Raw data of the airflow was digitized, monitored, and recorded by PowerLab/8sp (ADInstruments Inc., Colorado Springs, CO). Respiratory variables including $V_E$, $V_T$, $f_R$, and $T_E$ were averaged over a 10 min period and variables reflecting the variation of the respiratory intervals (SD1 and SD2) were derived from 2500 consecutive respiratory intervals during which the animal breathing was relatively stable. An apnea was defined as $T_E$ duration approximately three-fold 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$_1$R-IR alone and coupled with CASP3-IR or NP-IR were analyzed. NK$_1$R-IR was measured and averaged from the micrographs of the PBC as previously described (49). The co-localizations of NK$_1$R+NP-IR and NK$_1$R+CASP3-IR were expressed as the percentages (co-labeling area over the area with positive NK$_1$R-IR). All group data were reported as means ± SE or presented in box and whisker plots in some figures. Non-parametric (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-values < 0.05 were considered significant.
HK483 viral infection induced abnormal behaviors

At 2 dpi, the mice infected by HK483 or HK486 virus showed no discernible behavior abnormalities as compared to 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 1A 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 though it is 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 SD1 and SD2 at 2 and 4 dpi, but the variability became substantially enhanced later, especially SD1 at 6 dpi (Fig. 3A and 3B), 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 was associated with greatly increased apneic numbers and prolonged apneic duration (Fig. 4B). The longest apneic durations were 4.2 ± 1.1 s in HK483 mice, while these values were 1.4 ± 0.4 s and 1.2 ± 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 $pHa$, $PaO_2$ 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 animals were relatively lower as compared to the conscious state. Nevertheless, the difference of $PaO_2$ and $SaO_2$ between HK486 (Ctrl) and HK483 reflects the impacts of lethal H5N1 viral infection on these variables.

HK483 viral infection induced pulmonary edema compared to HK486 virus

We compared wet/dry weight ratios of the lungs and the brain at the four time points in the two infected groups. As listed in Table 1, compared to 0 dpi, HK486 failed to induce a significant change in both wet/dry weight ratios of the lungs and the brain at 2, 4, and 6 dpi, while HK483 viral infection significantly increased the wet/dry ratio of the lungs at 6 dpi.
HK483 virus replicated the PBC to cause apoptosis and reduce NK_1R neural population

Our immunohistochemical data illustrated in Fig 6 could be summarized as follows: (1) NP-IR was observed in the PBC, particularly local NK_1R neurons, only in HK483 mice. (2) PBC NK_1R-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 NK_1R-IR was reduced to 25% by HK483 virus compared to HK486 virus, about 61% of which showed co-labeling with NP-IR. (3) CASP3-IR was detected in the PBC, including NK_1R neurons, and 26% of the remaining 25% NK_1R-IR positive area was co-labeled 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) prior to 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 ventilatory 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 prior to death.

The mechanisms by which lethal H5N1 virus induces tachypnea at the early stage of the infection have not been determined, but it is 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, though further studies are warranted to provide the direct evidence.

PBC NK\(_1\)R 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 (14). The central infection by lethal H5N1 virus could induce neural apoptosis to lead to neural cytotoxicity and potentially to promote virus progeny (38). Staining of apoptosis in brain neurons was observed 4 dpi in lethal H5N1 infected mice (4, 13, 16). Thus, we tested whether the ataxic breathing and apneas observed at 6 dpi in HK483 mice were associated with viral replication in PBC NK\(_1\)R neurons and these neural apoptosis. Our results showed that HK483, but not HK486 virus, 1) replicated in the PBC, especially local NK\(_1\)R neurons; 2) induced apoptosis of these neurons; and 3) reduced NK\(_1\)R expression by 75%. In addition, PBC NK\(_1\)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\(_1\)R neurons against the viral infection-induced apoptosis prevents the lethality of H5N1 viral infection. The route by which the lethal H5N1 virus enters CNS 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:dry 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 BALF in HK483 than HK486 mice at 6 dpi (unpublished observation by Zhuang et al.). 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.

Acknowledgements

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37. Tankersley CG and Broman KW. Interactions in hypoxic and hypercapnic breathing are genetically linked to mouse chromosomes 1 and 5. *J Appl Physiol* 97: 77-84, 2004.


Figure Legends

Figure 1. Effects of H5N1 viral infection on body weight (BW), body temperature (BT), viral titer, and pulmonary inflammation/damage in mice. A: HK483, but not HK486, viral infection causes progressive BW loss (nearly 25% at 6 dpi). B: BT is not significantly affected at 2 dpi in all animals, but drops markedly thereafter only in HK483 mice. C: Viral titers in the lungs are similar between HK483 and HK486 mice at 4 and 6 dpi, but higher in the former than the latter at 2 dpi. Mean ± SE; n = 7, 7, 8, and 10 at Day 0, 2, 4, 6 in Ctrl, HK486, and/or HK483 mice respectively. * P < 0.05 compared 0 dpi; † P < 0.05 vs. Ctrl and/or HK486 at the given day; # P < 0.05 compared with the previous day in HK483 mice. D: The representative tissues of bronchial epithelium, alveoli, and vascular endothelium (three rows) collected at different time points (Day 0, 2, 4, 6) in HK486 and HK483 mice reflect a similar development of pulmonary inflammation/damage by the two types of viral isolations (bar = 20 µm).

Figure 2. Impacts of H5N1 viral infection on breathing. A: VE in HK486 mice is similar to Ctrl over the infection period; however, it is significantly increased at 2 and 4 dpi in HK483 mice and the increase returns to the pre-infection level at 6 dpi. B and C: The VE change is due to elevations in both fR and/or VT. Mean ± SE; n = 7, 7, 8, and 10 for Ctrl, HK486, and HK483 mice, respectively. * P < 0.05 vs. 0 dpi; † P < 0.05 compared to Ctrl and HK486 at the given day; # P < 0.05 vs. the previous day in HK483 mice.

Figure 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 (2500 intervals). Mean ± SE; n = 7, 7, 8, and 10 for Ctrl, HK486, and HK483 mice respectively. * P < 0.01 compared to 0 dpi; † P < 0.01 compared to Ctrl and HK486 at the given day; and # P < 0.05 vs. the previous day in HK483 mice.

Figure 4. Ataxic breathing at 6 dpi in HK483 mice. A: Typical recordings of resting breathing in a Ctrl, a HK486, and a HK483 mouse at 6 dpi. B: Corresponding group data of apnea numbers (left) and the averaged apneic duration (TE, right) in Ctrl, HK486, and HK483 mice; n = 7, 7, 8, and 10 for Ctrl, HK486, and HK483 mice respectively. * P < 0.01 compared to 0 dpi; † P < 0.01 compared to Ctrl and HK486.

Figure 5. Effects of H5N1 viral infection on arterial blood pH and gases. A: HK483, but not HK486, viral infection significantly lowers pH at 6 dpi. B-D: PaCO₂ starts to increase while PaO₂ and SaO₂ decrease at 4 dpi; these changes become greater at 6 dpi in HK483 mice. Mean ± SE; n = 7, 7, 8, and 10 for Ctrl, HK486, and HK483 mice at each time-point respectively. * P < 0.01 compared to 0 dpi; † P < 0.01 compared to Ctrl and HK486 at the given day; # P < 0.05 vs. the previous day in HK483.

Figure 6. PBC immunoreactivity (IR) of NK1R, NP, CASP3 alone and NK₁R co-localization with NP or CASP3 in HK483 and HK486 mice at 6 dpi. A: Typical micrographs from the PBC. NK₁R-IR, but not NP-IR (the first column) and CASP3-IR (the second column), is detectable in the PBC of a HK486 mouse. In sharp contrast, a remarkable expression of NP-IR (the third
column) and CASP3-IR (the fourth column) alone and coupled with NK₁R is observed in a HK483 mouse. The inset with higher power images more clearly shows the apoptotic NK₁R neurons. B: left, middle, and right panels show group data of PBC NK₁R-IR optical density, ratio of co-localized positive NP-IR in NK₁R areas, and ratio of co-localized positive CASP3-IR in NK₁R areas, respectively; n = 5 and 5 for HK486 and HK483 mice. * P < 0.01 for HK483 vs. HK486. Scale bar = 50 μm. Note: the values from HK486 mice in the middle and right panels are zero.
Table 1. Dry/wet weight ratios of lung and brain tissues (Mean ± SE)

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* P < 0.05, 6 dpi vs. 0 dpi