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Review

The neuropathogenesis of highly pathogenic avian influenza H5Nx viruses in mammalian species including humans

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Circulation of highly pathogenic avian influenza (HPAI) H5Nx viruses of the A/Goose/Guangdong/1/96 lineage in birds regularly causes infections of mammals, including humans. In many mammalian species, infections are associated with severe neurological disease, a unique feature of HPAI H5Nx viruses compared with other influenza A viruses. Here, we provide an overview of the neuropathogenesis of HPAI H5Nx virus infection in mammals, centered on three aspects: neuroinvasion, neurotropism, and neurovirulence. We focus on *in vitro* studies, as well as studies on naturally or experimentally infected mammals. Additionally, we discuss the contribution of viral factors to the neuropathogenesis of HPAI H5Nx virus infections and the efficacy of intervention strategies to prevent neuroinvasion or the development of neurological disease.

Highlights

Highly pathogenic avian influenza (HPAI) H5Nx viruses can cause neurological complications in many mammalian species, including humans.

Neurological disease induced by HPAI H5Nx viruses in mammals can manifest without clinical respiratory disease.

HPAI H5Nx viruses are more neuropathogenic than other influenza A viruses in mammals.

Severe neurological disease in mammals is related to the neuroinvasive and neurotropic potential of HPAI H5Nx viruses.

Cranial nerves, especially the olfactory nerve, are important routes of neuroinvasion for HPAI H5Nx viruses.

HPAI H5Nx viruses have a broad neurotropic potential and can efficiently infect and replicate in various CNS cell types.

Vaccination and/or antiviral therapy might in part prevent neuroinvasion and neurological disease following HPAI H5Nx virus infection, although comprehensive studies in this area are lacking.

Emergence and circulation of H5Nx viruses

HPAI H5Nx viruses of the A/Goose/Guangdong/1/96 (Gs/Gd) lineage emerged more than 25 years ago and were first isolated from domestic geese in 1996 [1]. Shortly thereafter, the first human infection was documented [2], and since then, a total of 868 human cases have been detected, of which 457 were fatal[i]. The phylogenetic tree of the Gs/Gd lineage is based on the sequence of the trimeric surface protein hemagglutinin (HA)[ii] [3]. The extensive circulation and continuous evolution of H5N1 viruses led to a diversification of the HA protein, resulting in multiple clades and subclades, of which subclade 4.3.3.2b is currently spreading worldwide via wild birds [4,5]. Additionally, **reassortment** (see Glossary) events have occurred, which led to the exchange of non-HA gene segments between viruses. This resulted in local circulation of, for example, H5N6 or H5N8 viruses, which are all descendants of the ancestral Gs/Gd isolate that emerged in 1996 [6–9]. In this review, we refer to the Gs/Gd lineage viruses as HPAI H5Nx viruses.

Prior to 2005, HPAI H5Nx viruses were predominately circulating in poultry species in Asia, with incidental spillovers to migratory birds resulting in local outbreaks without sustained transmission within wild birds year-round [9]. Since 2021, HPAI H5Nx viruses, specifically from clade 2.3.4.4b, have been circulating continuously in wild birds with intercontinental detections in Asia, Africa, Europe, North America, and South America, resulting in mass mortalities in different wild bird species [10,11]. In addition, HPAI H5Nx viruses are frequently detected in mammalian species that feed on sick or dead infected birds. So far, mammal-to-mammal transmission seems rare but has been suggested in 2003 among tigers [12,13] and recently in minks [14] and sea lions [15].

Neuropathogenesis of H5Nx viruses

A unique feature of HPAI H5Nx viruses is their ability to cause severe neurological disease in birds and mammals, a feature rarely observed for other influenza A viruses. Neurological complications

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are the most common clinical manifestations in naturally infected mammals, although one could argue that this may be due to the ease of observation of neurological versus respiratory symptoms. However, studies from experimentally infected mammals show that neurological complications occur more frequently after HPAI H5Nx virus infection than infection with low pathogenic avian influenza (LPAI), seasonal or pandemic influenza A viruses [16,17]. To date, neurological disease has been reported in many mammalian species, including humans, foxes, cats, tigers, stone martens, harbor porpoises, common seals, gray seals, ferrets, mice, pikas, and minks [11]. In this review, we discuss current knowledge on the neuropathogenesis of HPAI H5Nx virus infections in mammals, focusing on three aspects: **neuroinvasion**, **neurotropism**, and **neurovirulence**. Although we focus on mammals, we summarize the neuropathogenic potential of these HPAI H5Nx viruses in birds in Box 1. Last, we outline the role of viral factors and how these contribute to neuropathogenicity of HPAI H5Nx viruses in mammals and possible intervention strategies and treatment options in humans.

Neuroinvasion

'Neuroinvasion' refers to the ability of a virus to enter the PNS or CNS [18]. Influenza A viruses initially infect cells within the respiratory tract and from there can spread to the CNS. Possible pathways include virus transport within or along cranial nerves (CNs) (Figure 1A,B) or hematogenous spread, after which it may cross the blood–brain barrier (BBB) or blood–cerebral spinal fluid barrier (BCSFB). CNs that innervate the mammalian respiratory tract include the olfactory nerve (CN I; sensory fibers) and trigeminal nerve (CN V; sensory and motor fibers) in the nasal cavity, the facial nerve (CN VII; sensory, parasympathetic and motor fibers) and glossopharyngeal nerve (CN IX; sensory and motor fibers) in the upper respiratory tract, and the vagus nerve (CN X; sensory, parasympathetic and motor fibers) in the lower respiratory tract. Evidence suggests that HPAI H5Nx viruses can use the olfactory [16,19–30], trigeminal [22–24,27,30,31], facial [28,31], vestibulocochlear [19,22,27], vagus [22–24], and upper thoracic sympathetic nerves [23] to enter the CNS in mammals, as virus antigen is detected in the soma and/or axons of sensory neurons of these nerves (Figure 1C).

The nasal cavity is lined by respiratory and olfactory mucosa. The surface ratio of respiratory and olfactory mucosa differs between mammalian species. In humans, it is estimated that around

Box 1. The neuropathogenic potential of HPAI H5 viruses in birds

Neurological complications are regularly observed in birds infected with HPAI H5Nx viruses. Many species develop neurological signs, including chickens and turkeys (Galliformes), ducks and geese (Anseriformes), and raptors (Falconiformes) [167–174]. The neuropathogenicity of HPAI H5Nx viruses differs among avian species. Infection starts in the respiratory tract and/or gastrointestinal tract, after which the virus can spread to other tissues including the CNS [174]. The route of neuroinvasion is not well studied in birds. In gallinaceous poultry, HPAI H5Nx viruses have a strong endotheliotropism, which is not observed in wild birds such as mallard ducks [174]. Infected damaged endothelial cells may lead to hemorrhages, edema, congestion, and thrombosis [174] and form a likely route of hematogenous virus spread to the CNS in gallinaceous poultry species. The route of neuroinvasion in bird species without evident endotheliotropism such as ducks remains to be unraveled. Inside the CNS, various cells of the CNS, including ependymal cells, glial cells, and neurons, may become infected, demonstrating the virus' neurotropism. Infected cells produce progeny virus and become necrotic, which triggers infiltration of inflammatory cells, gliosis, hemorrhages, and edema [174,175], consistent with encephalitis. Systemic spread and multiorgan virus infection usually induces similar acute necrohemorrhagic lesions, most notably within lungs, heart, pancreas, and liver. If evident, macroscopic CNS lesions may consist of malacia, hemorrhages, and edema [174,175]. Neurological signs commonly reported include head twitching, ataxia, tremors, torticollis, and opisthotonos [174,175]. The degree of neurovirulence of HPAI H5Nx viruses depends on the virus and the infected bird species. For example, natural infection in farmed domestic ducks was shown to result in more severe neurological disease compared with wild mallard ducks [175]. Furthermore, HPAI H5Nx infection in raptors such as buzzards, falcons, and sea eagles is associated with severe, often fatal, neurological disease [169,170,172,173]. Since the global spread of HPAI H5Nx viruses, a wider range bird species has been infected, which resulted in mass mortality in sensitive bird species [176]. Furthermore, the carcasses of infected birds form a source of infection for opportunistic scavenging terrestrial and aquatic carnivores.

Glossary

Neuroinvasion: the ability of a virus to enter either the PNS or CNS.
Neurotropism: the ability of a virus to infect and replicate in cells of the nervous system. Cells of the nervous system include neurons, glial cells (e.g., astrocytes, oligodendrocytes, oligodendrocyte precursor cells, microglia), meningeal cells, choroid plexus cells, and cells of the neurovascular system (such as endothelial cells and pericytes).
Neurovirulence: the ability of a virus infection to cause damage in the CNS that contributes to the development of clinical disease of the nervous system independently of the ability of the virus to invade the CNS and infect cells of the CNS.
Reassortment: a characteristic of multisegmented RNA viruses; refers to the exchange of viral gene segments in cells coinfected with at least two different viruses to produce virus progeny with a novel genome combination.
Synaptic plasticity: refers to the ability of synaptic connections to strengthen or weaken over time, which is an important neurophysiological process supporting learning, memory, and other cognitive functions.

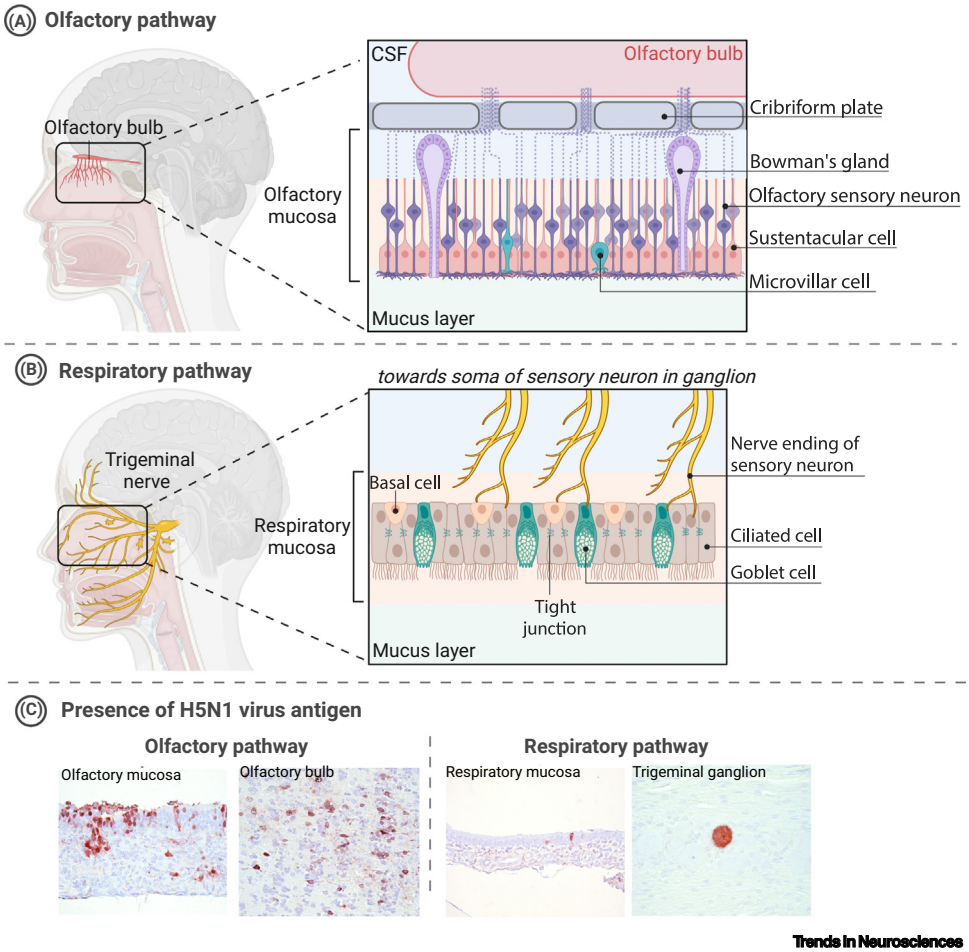

Figure 1. Routes of neuroinvasion for highly pathogenic avian influenza (HPAI) H5Nx viruses. (A) The olfactory pathway includes the olfactory mucosa, which contains olfactory sensory neurons. The ciliated neurons extend into the nasal cavity, and their axons pass through the cribriform plate and terminate in the olfactory bulb in the brain. (B) The respiratory pathway includes the respiratory mucosa. Nerve endings of sensory neurons reside behind the tight junctions of epithelial cells and are not in direct contact with the environment. Their cell bodies are found in ganglia. (C) The presence of H5N1 virus nucleoprotein antigen is shown in the olfactory pathway and the respiratory pathway in experimentally infected ferrets. Images adjusted from [30]. Figure created with Biorender.

1.25% of the nasal mucosa consists of olfactory mucosa (2–10 cm^2) [32,33], while in mice this is around 47% [34]. The olfactory mucosa is responsible for odor recognition and comprises olfactory sensory neurons (OSNs), sustentacular cells, microvillar cells, basal cells, olfactory ensheathing cells, and cells of the Bowman's glands [32]. The dendrites of the bipolar OSN have cilia that are directly exposed to the environment (Figure 1A) [35,36]. Neuroinvasion along the olfactory nerve starts by viral infection of cells within the olfactory mucosa. Several studies in mice and ferrets have shown that HPAI H5Nx viruses replicate efficiently in the olfactory mucosa with virus antigen in all cell types, including OSN [20,29,30]. Infection of OSN can result in anterograde transport of virus through the cribriform plate of the ethmoid bone into the olfactory bulb, the brain structure involved in olfaction. In the olfactory bulb, axons of the OSN synapse in glomeruli with mitral cells and periglomerular cells in the glomerular layer, which have been found infected in experimentally inoculated ferrets [20,29]. Alternatively, viruses could diffuse through

continuous, fluid-filled channels created by olfactory ensheathing cells along the olfactory nerve that are filled with CSF and end up in the meninges [25]. Efficient replication of HPAI H5Nx viruses in the olfactory mucosa likely contributes to this propensity for neuroinvasion, as seasonal and pandemic influenza viruses replicate less efficiently in the olfactory mucosa and are less frequently associated with neuroinvasion along the olfactory nerve [16,37]. Transmission of HPAI H5N1 virus along the olfactory nerve does not result in a genetic bottleneck [30].

Within the respiratory mucosa, nerve endings of the sensory neurons are located directly below the tight junctions of the respiratory epithelial cells and, unlike OSN endings, are not in direct contact with the environment (Figure 1B). The nerve fibers of the trigeminal nerve innervate, among others, the respiratory mucosa of the nasal cavity, the nasopharynx, the sinuses, and the palate. CNS invasion along the trigeminal nerve has been observed in experimentally inoculated mice and ferrets, where virus antigen was detected in the trigeminal ganglion [20,23,24,29,38]. Evidence for neuroinvasion of HPAI H5Nx viruses along the facial nerve is based on the detection of virus antigen in the facial nucleus and solitary nucleus in experimentally inoculated mice and ferrets [22,28,31]. The vestibulocochlear pathway has been implicated in CNS invasion in ferrets and mice. In ferrets, HPAI H5Nx virus antigen was detected in epithelial cells of the eustachian tube [27] and in the cochlea and vestibulocochlear nerve [22]. In intranasally inoculated mice, virus antigen was detected in vestibulocochlear nuclei [22]. Neuroinvasion along the vagus nerve, of which sensory nerve terminals are widely distributed throughout the lower respiratory tract, has been shown in mice [28,38,39].

Hematogenous spread of HPAI H5Nx viruses could result in subsequent CNS entry through the BBB or BCSFB, but there is little evidence that HPAI H5Nx viruses invade the CNS via this route. HPAI H5Nx viruses can spread to the circulation (viremia) in both humans and experimentally inoculated animals [40–43]. In contrast, viremia is not commonly observed during seasonal influenza virus infections in mammals [42,44,45]. To our knowledge, there is currently no evidence for trans- or paracellular transport of cell-free or cell-associated HPAI H5Nx virus over the BBB or BCSFB. However, in intragastrically inoculated cats and naturally infected foxes, HPAI H5Nx viruses have been shown to infect few endothelial cells in the CNS, from where viruses could spread across the BBB [46,47]. Of note, although in poultry and black swans, endothelial cell infection with HPAI H5Nx viruses is a common observation, endothelial infection in mammals appears to be rare.

Neurotropism

Neurotropism is the ability of a virus to infect cells of the CNS and replicate in them [18]. Once the virus invades the CNS, a variety of cell types in the brain can become infected, such as neurons, glial cells (astrocytes, microglia, oligodendrocytes, and ependymal cells), choroid plexus cells, neural endothelial cells, and pericytes. The infection efficiency of HPAI H5Nx viruses varies among the different CNS cell types.

The surface protein HA facilitates attachment to and entry into susceptible cells. The receptor binding properties of HA determine in part the cell tropism. Avian viruses, for example, preferentially bind to α(2,3)-linked sialic acids (SIAs), whereas human influenza viruses recognize α(2,6) SIAs (reviewed in [48]). While the presence of SIAs in the respiratory tract is well studied [49–51], their presence in different anatomical locations of the mammalian CNS is largely uncharacterized. The CNS has a high SIA content, and several studies suggest the presence of both α(2,3)- and α(2,6)-linked SIAs in mammalian species such as mice, pigs, cats, dogs, and humans (Table 1). SIA distribution in the CNS of ferrets, one of the most widely used animal models for influenza research, has not been investigated, to our knowledge.

Table 1. Distribution of sialic acids in CNS of various mammals[a,c]

Species	α(2,3)-Sialic acids (MAA)		α(2,6)-Sialic acids (SNA)		Refs
	Areas	Cell type	Areas	Cell type	
Mouse	Olfactory bulb: Yes Hippocampus: Yes Thalamus: Yes Cerebellum: Yes Pons: No Medulla oblongata: No	N, VE, E	Olfactory bulb: Yes Hippocampus: Yes Thalamus: Yes Cerebellum: Yes Pons: Yes Medulla oblongata: Yes	N, G, VE, E	[128]
	Cerebrum: No Cerebellum: Yes	–[b]	Cerebrum: No Cerebellum: Yes	–	[129]
	Substantia nigra: Yes Hippocampus: Yes Cerebellum: Yes	–	Substantia nigra: No Hippocampus: No Cerebellum: No	–	[130]
Human	Cerebral cortex: Yes Hippocampus: Yes Brain stem: Yes Cerebellum: Yes	N, G, VE, E	Cerebral cortex: Yes Hippocampus: Yes Brain stem: Yes Cerebellum: Yes	N, G, VE, E	[128]
	–	N, VE	–	VE	[131]
Pig	–	N	–	VE	[132]
Cat	Cerebellum: Yes		Cerebellum: Yes		[133]
Dog	–	VE	–	VE	[134]

[a]It should be noted that experimental variation between different lectin suppliers is observed and needs critical evaluation [135].
[b]–, data not available.
[c]Abbreviations: E, ependymal cells; G, glial cells; N, neurons; VE, vascular endothelial cells.

In vitro and *in vivo,* HPAI H5Nx viruses can infect many different CNS cell types. *In vitro* studies showed that HPAI H5Nx viruses infect and replicate in neuron- and astrocyte-like cells [52,53], in primary mouse astrocytes and microglia [54,55], and in different human induced pluripotent stem cell (hiPSC)-derived cell types such as neural progenitor cells, neurons, and astrocytes [56]. These *in vitro* studies show efficient infection of CNS cells, based on increasing viral titers in the supernatant over time. In experimentally inoculated ferrets, mice, and pikas and naturally infected red foxes, cats, tigers, stone martens, harbor porpoises, common seals, and gray seals, both neurons and glial cells were infected with HPAI H5Nx virus, based on the detection of virus antigen (Tables 2 and 3) [13,20,23–28,57–72]. Occasionally, infected ependymal, choroid plexus, and meningeal cells were observed [19,20,27,61,62,65,71,73]. The detection of virus antigen in different anatomical locations and the isolation of infectious virus from them (Figure 2) indicate that HPAI H5Nx viruses replicate efficiently within the CNS of mammals [16,74]. However, the replication efficiency and distribution within the CNS seem to be strain dependent. For instance, while A/HK/483/97 is known to disseminate throughout the CNS, A/HK/486/97 does not exhibit the same level of spread, despite both strains being able to invade the CNS [75]. The neurotropism in humans is poorly studied as postmortem CNS samples are often not collected for various reasons. However, in the few cases described, influenza virus antigen was detected in neurons and glial cells in various areas of the brain such as the cerebral cortex, hippocampus, midbrain, and cerebellum (Table 4) [76–78].

Taken together, after neuroinvasion, HPAI H5Nx viruses can infect and replicate efficiently in various CNS cell types in different mammalian species. Depending on the route of neuroinvasion, various cells become exposed, after which the virus possibly disseminates to other regions of the CNS. The mechanism of HPAI H5Nx virus transmission between cells of the CNS is not well studied, but it has been shown that transport of HPAI H5Nx virus can occur through transaxonal

Table 2. Experimentally HPAI H5Nx virus-inoculated mammals in which neuroinvasion and/or neurovirulence has been observed[b,c]

Strain	Species	Neuroinvasion	Neurotropism	Neurovirulence			Refs
				Inflammatory response	CNS lesions	Neurological signs	
A/Hong Kong/156/97	Macaques	Yes[a]	No	No	No	No	[136]
	Mice	Yes[a]	N, G	–[d]	Yes	–	[24,59]
A/Hong Kong/483/97	Mice	Yes, ON, TN, VN	N, G, S, E	Yes	Yes	Yes	[23,24,59,68,75,118,137]
	Ferret	Yes, ON	No	–	Yes	Yes	[31,68,137,138]
A/HongKong/486/1997	Mice	Yes[a]	–	–	–	No	[68,137]
	Ferret	Yes[a]	–	–	Yes	Yes	[68,137,138]
A/chicken/Hong Kong/YU822.2/2001	Mice	Yes[a]	–	–	Yes	Yes	[139]
A/chicken/Hong Kong/YU562/2001	Mice	No	–	–	–	–	[139]
A/pheasant/Hong Kong/FY155/2001	Mice	Yes[a]	–	–	–	Yes	[139]
A/chicken/Hong Kong/FY150/2001	Mice	Yes[a]	–	–	–	Yes	[139]
A/chicken/Hong Kong/NT873.3/2001	Mice	Yes[a]	–	–	–	Yes	[139]
A/Hong Kong/213/03	Ferret	Yes[a]	–	–	–	–	[69]
A/Chicken/Indonesia/7/03	Mice	No	–	–	–	No	[26,68]
	Ferret	No	–	–	–	–	
A/Chicken/Vietnam/NCVD/8/2003	Mice	No	–	–	–	–	[68]
A/Chicken/Korea/ES/2003	Mice	No	–	–	–	No	[68]
	Ferret	Yes[a]	–	–	–	No	
A/Vietnam/1194/2004	Cats	Yes[a]	N, G, E	–	Yes	No	[61]
A/Vietnam/1203/04	Mice	Yes, ON, VN, FN	N, G, E	Yes	Yes	Yes	[26,28,65,68,88]
	Ferrets	Yes, ON, VON, H	N, G, E	–	Yes	Yes	[19,26,27,31,68,69,74]
	Hamsters	Yes[a]	–	–	–	–	[74]
A/Vietnam/1204/04	Mice	Yes[a]	–	–	–	No	[68]
	Ferrets	Yes[a]	N	–	Yes	Yes	
A/Vietnam/UT3062/04	Ferret	Yes[a]	–	–	–	–	[74]
	Hamsters	Yes[a]	–	–	–	–	
A/Thailand/16/2004	Mice	Yes[a]	–	–	–	No	[68]
	Ferrets	Yes[a]	–	–	Yes	No	
A/Thailand/SP/83/2004	Mice	No	–	–	–	No	[68]
	Ferrets	Yes[a]	–	–	No	No	
A/Thailand/Kan/353/2004	Ferret	Yes[a]	–	–	Yes	Yes	[68]
A/Chicken/Vietnam/NCVD/31/2004	Mice	No	–	–	–	No	[68]
	Ferrets	Yes[a]	–	–	No	No	
A/birds/Qinghai/07/04	Mice	Yes[a]	–	–	Yes	No	[140]
A/Whooper swan/Mongolia/244/05	Mice	Yes[a]	N.S.	–	Yes	–	[26]
	Ferrets	Yes[a]	–	–	–	No	
A/Muscovy duck/Vietnam/209/05	Mice	Yes[a]	N.S.	–	Yes	Yes	[26]
	Ferret	–	–	–	–	No	
A/Vietnam/JP36-2/05	Ferret	Yes[a]	N	–	Yes	Yes	[69]
A/Indonesia/5/2005	Ferrets	Yes, ON	N, G, E	–	Yes	Yes	[20,25]

Table 2. (continued)

Strain	Species	Neuroinvasion	Neurotropism	Neurovirulence			Refs
				Inflammatory response	CNS lesions	Neurological signs	
A/goose/Krasnoozerskoye/627/05	Mice	Yes[a]	–	Yes	–	–	[141]
A/turkey/Suzdalka/12/05	Mice	Yes[a]	–	–	–	–	[141]
A/Turkey/65-596/06	Ferret	Yes[a]	–	–	No	No	[69]
A/duck/Tuva/01/06	Mice	Yes[a]	–	–	–	–	[141]
A/chicken/Krasnodar/123/06	Mice	Yes[a]	–	–	–	–	[141]
A/chicken/Reshoty/02/06	Mice	Yes[a]	–	–	–	–	[141]
A/whooper swan/Germany/R65-1/2006	Red fox	Yes[a]	N, G	–	Yes	No	[66]
A/great black-headed gull/Qinghai/1/2009	Pika	Yes[a]	N	–	Yes	–	[57]
A/Vietnam/HN36285/2010	Cats	Yes[a]	N.S.	–	Yes	No	[142]
	Dogs	No	-	–	No	No	
A/crow/India/02CA01/2012	Mice	Yes[a]	-	–	Yes	-	[63]
A/black-headed gull/Netherlands/29/2017 (H5N6)	Ferret	Yes, ON, TN	N, G, E	–	Yes	No	[143]

[a]Route of neuroinvasion is not specified.
[b]All virus strains are H5N1 viruses unless otherwise indicated. Neurotropism is defined as positive signal IHC/ISH in defined cell type.
[c]Abbreviations: E, ependymal cells; FN, facial nerve; G, glial cells; H, hematogenous; N, neuron; N.S.; cell type not specified; ON, olfactory nerve; S, Schwann cells; TN, trigeminal nerve; VN, vagus nerve; VON, vestibulocochlear nerve.
[d]–, data not available.

transport within axons of primary dorsal root ganglia neurons; yet, transsynaptic transmission of viruses was not shown [28].

Neurovirulence

'Neurovirulence' refers to the ability of the virus infection to cause damage in the CNS that contributes to the development of clinical disease, independent of the neuroinvasive and neurotropic potential of the virus [18]. The neurovirulence of HPAI H5Nx virus infection is predominantly observed and studied in cases where virus entered and spread through the CNS. However, HPAI H5Nx virus infections might also trigger neurological complications in the absence of neuroinvasion. Neurological signs in HPAI H5Nx virus-infected mammals include ataxia, tremors, convulsions, paralysis, and seizures and are observed in many mammalian species, including cats, tigers, and foxes [13,47,60,67,73,79,80]. Whether there are differences in the (neuro)pathogenesis among infected mammals is currently unknown (Tables 2 and 3).

In naturally infected mammals, neurological signs following HPAI H5Nx virus infection are often observed, which might relate to the attention these animals attract due to atypical behaviors such as ataxia, heightened aggression, or lack of fleeing behavior (Table 3). In humans, neurological symptoms vary considerably, ranging from mild headache to severe symptoms such as seizures and convulsions (Table 4). *In vivo* studies show that HPAI H5Nx neurovirulence is determined in part by the route and dose of inoculation, as well as virus strain and infected animal species (Table 2). For example, inoculation with A/Muscovy-duck/Vietnam/209/05 resulted in neurological signs in mice, but not in ferrets [26]. However, what proportion of infections results in severe neurological signs and how this specifically differs among mammalian species and virus isolates are currently unknown (Figure 3).

Table 3. Naturally HPAI H5Nx virus-infected mammals in which neuroinvasion and/or neurovirulence has been observed[b,c]

Year(s) and location of infection	Species	Neuroinvasion	Neurotropism	Neurovirulence		Refs
				CNS lesions	Neurological signs	
2003 – Thailand	Tiger, leopard	–[d]	–	Yes	–	[12]
2004 – Thailand	Cat	Yes[a]	N	Yes	Yes	[67]
2004 – Thailand	Dog	No	–	No	–	[144]
2004 – Thailand	Tiger, leopard	Yes[a]	N	Yes	Yes	[13]
2005 – Vietnam	Civet	Yes[a]	N	Yes	Yes	[145]
2006 – Germany	Cat	Yes[a]	–	–	–	[146]
2006 – Germany	Stone marten	Yes[a]	N, G	Yes	Yes	[70]
2013 – China	Tiger	Yes[a]	–	N.A.	Yes	[147]
2014/2015 – China	Tiger	–	–	Yes	Yes	[148]
2016/2017 – South Korea (H5N6)	Cat	Yes[a]	N, G, E	Yes	Yes	[73]
2020 – United Kingdom (H5N8)	Seals	Yes[a]	N	Yes	Yes	[71]
	Red fox		N, E		No	
2021 – The Netherlands	Red fox	Yes[a]	N.A.	–	Yes	[79]
2021 – Germany (H5N8)	Harbor seal	Yes[a]	N, G	Yes	–	[149]
2021/2022 – Finland	Otter	Yes[a]	–	Yes	Yes	[150]
	Red fox			No	–	
	Lynx			N.A.		
2021/2022 – The Netherlands	Red fox	Yes[a]	N, G	Yes	Yes	[60]
2021/2022 – The Netherlands	Red fox, polecat, badger, otter	Yes[a]	N	Yes	Yes	[80]
2022 – Sweden	Porpoise	Yes[a]	N, G, E	Yes	Yes	[72]
2022 – Canada	Red fox	Yes[a]	N.A.	Yes	Yes	[47]
	Mink, skunk	–		–		
2022 – USA	Seals	–	–	–	Yes	[151]
2022 – Spain	Mink	–	–	–	Yes	[14]
2023 – Peru	Sea lion	–	–	–	Yes	[152]
2023 – Chile	Sea lion	–	–	Yes	Yes	[15]
2023 – USA	Dolphin	Yes[a]	N	Yes	–	[153]

[a]Route of neuroinvasion is not specified.
[b]All isolated viruses are H5N1 viruses unless otherwise indicated. Neurotropism is defined as positive signal IHC/ISH in defined cell type.
[c]Abbreviations: E, ependymal cells; G, glial cells; N, neuron; N.S., cell type not specified; S, Schwann cells.
[d]–, data not available.

The neurovirulence of HPAI H5Nx virus infection is associated with virus entry into and spread throughout the CNS, as well as the resulting damage and inflammation. Infection of CNS cells can result in cell death (via necrosis and/or apoptosis) and can incite an inflammatory response that can lead to a dysregulated neuronal homeostasis. Inflammation is typically characterized by induction of proinflammatory cytokines and chemokines, infiltration of inflammatory cells, activation of glial cells (gliosis), and edema. Malacia and hemorrhage may be present in severe inflammatory lesions. Such acute lesions in HPAI H5Nx virus-infected mammals typically colocalize with the presence of virus antigen and are consistent with a diagnosis of viral encephalitis or meningoencephalitis [25,30].

Cell death of HPAI H5Nx virus-infected CNS cells has been observed *in vitro* and *in vivo*. *In vitro*, apoptosis was induced by HPAI H5Nx viruses in human astrocyte-like cells and primary mouse

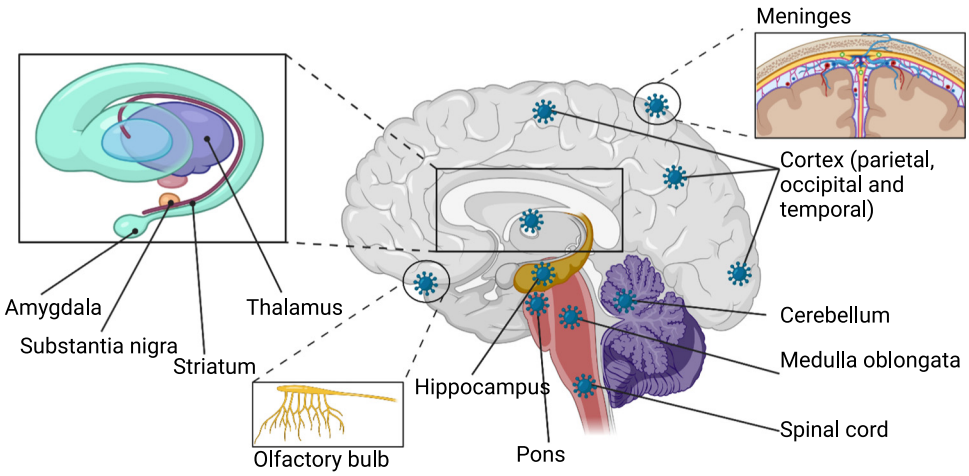

Figure 2. Anatomical locations with confirmed highly pathogenic avian influenza (HPAI) H5Nx virus detection in the mammalian brain. HPAI H5Nx viruses are detected in different anatomical locations within the brain. Here, all locations in which virus antigen has been detected in experimentally or naturally infected mammals are projected on a schematic overview of the human brain. Figure created with Biorender.

microglia and astrocytes [53,55]. Apoptosis might be triggered by upregulation of genes involved in interferon (IFN)-α/β signaling, Toll-like/RIG-I-like receptor signaling pathways, or elevation of internal Ca^{2+} in human astrocyte-like cells [53]. *In vivo*, HPAI H5Nx virus infection triggers neuronal degradation and cell death [20,29].

Infection of various brain cells with HPAI H5Nx virus elicits an immune response that can cause dysregulation of the brain homeostasis. These immune responses can trigger influx of immune cells to the site of inflammation or dysregulated neuronal plasticity by changing synapse structure and function [81,82]. *In vitro* HPAI H5N1 infection of neuron-like cell lines, hiPSC-derived neural models, or primary CNS cells results in the induction of proinflammatory cytokines such as type I IFN, type III IFN, IFN-γ-induced protein 10 (IP-10), interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor-α (TNF-α) [53,55,56,83,84]. *In vivo*, experimental infection of ferrets with influenza A viruses induced TNF-α, IL-6, and IL-8 in endothelial cells, neurons, and glial cells in the CNS [85–87]. Cell and tissue damage colocalizes with an influx of inflammatory cells, which may include monocytes, macrophages, lymphocytes, plasma cells, and neutrophils [20,24,29]. Furthermore, HPAI H5Nx virus infection elicits a prominent IP-10 response in the CNS [28,88], which is a chemoattractant for immune cells such as monocytes, macrophages, and dendritic cells and weakens the integrity of the BBB [89]. However, how each of these chemokines and cytokines contribute to the development of neuroinflammation or clinical disease is not fully understood, even though it is known that, for example, type I IFN can cause headaches [90].

Additionally, influenza A virus infections can disturb the brain homeostasis through alterations in hippocampal neuron morphology and neuronal connectivity [91]. This can be attributed to virus replication within CNS cells but also to local immune responses. Cytokines such as IL-1β, IL-6, and TNF-α emerge as important cytokines that disrupt the baseline physiology of synapses [82]. Unlike IL-6, which dampens neural activity in the CA1 region of the hippocampus [92], IL-1β and TNF-α can enhance excitatory as well as inhibitory neurotransmission through decreasing or enhancing the flow of Na^+, K^+, or Ca^{2+} ions [93–98]. All three cytokines have been linked to seizures and memory and learning deficits in various models of epilepsy, multiple sclerosis, and neurodegeneration [99–102]. HPAI H5Nx virus infection in an astrocyte-like cell line changed

Table 4. Human cases of HPAI H5Nx virus infection in which neuroinvasion and/or neurovirulence has been observed[c,d]

Year(s) and location of infection	Age, sex	Neuroinvasion	Neurotropism	Neurovirulence		Outcome	Refs
				CNS lesions	Neurological symptoms		
1997 – Hong Kong	13F	No	–	Yes	Yes	Fa	[154,155]
	25F	–[e]				Fa	
	24F			–		R	
2003/2004 – Thailand	6M	Yes	–	Yes[a]	No	Fa	[156,157]
2004 – Vietnam	4M	Yes	–	–	Yes	Fa	[158]
2004/2005 – Vietnam, Thailand, Cambodia	5 patients[b]	–	–	–	Yes	–	[159]
2005 – Indonesia	8F	–	–	–	Yes	Fa	[160]
	21M					R	
2005 – Cambodia	4 patients[b]	–	–	–	Yes	–	[161]
2005 – Ho Chi Minh City	1 patient[b]	–	–	–	Yes	–	[159]
2005 – China	24F	–	–	–	Yes	Fa	[77]
	35M	Yes	N	No			
2005/2008 – China	4 patients[b]	–	–	–	Yes	–	[162]
2005/2006 – Indonesia	7 patients[b]	–	–	–	Yes	–	[159]
2005/2006 – Turkey	1 patient[b]	–	–	–	Yes	–	[163]
2006 – Azerbaijan, Turkey	7 patients[b]	–	–	–	Yes	–	[159]
2006/2007 – Egypt	19 patients[b]	–	–	–	Yes	–	[159]
2008 – China	42M	Yes	N, G	Yes	No	Fa	[76]
2012 – China	2M	Yes	–	Yes	Yes	R	[164]
2013 – Canada	28F	Yes	–	Yes	Yes	Fa	[165]
2022 – China (H5N6)	6F	Yes	–	Yes	Yes	R	[166]

[a]Also induction of proinflammatory cytokines.
[b]Age and sex unknown.
[c]All isolated viruses are H5N1 viruses unless otherwise indicated. Neurotropism is defined as positive signal IHC/ISH in defined cell type.
[d]Abbreviations: E, ependymal cells; F, female; Fa, fatal; G, glial cells; M, male; N, neuron; N.S., cell type not specified; R, recovered; S, Schwann cells.
[e]–, data not available.

expression profiles of genes associated with neuroactive ligand–receptor interaction and internal Ca^{2+}, important for neuron-to-neuron signaling transduction and **synaptic plasticity** [53]. Nonstructural protein 1 (NS1) of HPAI H5Nx virus interacts with postsynaptic density protein 95 (PSD95), which reduces nitric oxide (NO) levels [103]. *In vivo* studies in mice described accumulation of α-synuclein in the olfactory bulb, hippocampus, locus coeruleus, and solitary nucleus up to 90 days after infection [28]. These data together suggest that HPAI H5Nx viruses disrupt neural homeostasis, which might contribute to the development of neurodegenerative diseases.

To summarize, HPAI H5Nx viruses, unlike seasonal or pandemic influenza A viruses, are highly neurovirulent in many mammalian species. The neurovirulence caused by the high neuroinvasive and neurotropic potential of HPAI H5Nx viruses, together with the induction of systemic cytokines, can contribute to the development of severe neurological signs, including behavior changes such as lack of fleeing behavior and aggression. Several studies suggest that HPAI H5Nx virus infection can result in milder CNS complications when the virus does not enter the CNS or when virus replication within the CNS is quickly controlled, but how this modulates behavioral outcomes or neurological symptoms is not well understood. The exact types and frequencies of neurological complications associated with HPAI H5Nx infection as well as the

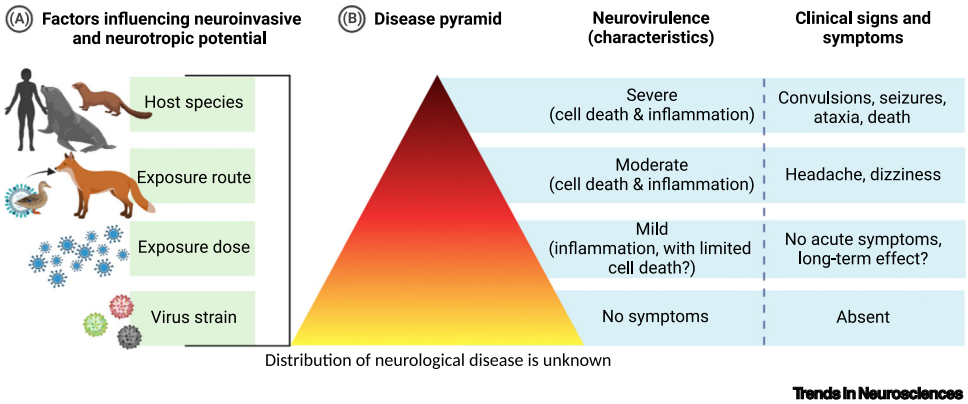

Figure. 3. Disease pyramid of the neurological signs associated with highly pathogenic avian influenza (HPAI) H5Nx virus infections in mammals. (A) Factors that influence neuroinvasion and neurotropism of HPAI H5Nx viruses. (B) The distribution of neurological disease and symptom severity following HPAI H5Nx virus infection is largely unknown. The neurovirulence of the virus can be broadly divided into four categories, ranging from no symptoms to severe symptoms. Figure created with Biorender.

temporal and spatial kinetics of the neurovirulence of HPAI H5Nx viruses are not fully understood (see Figure 3 and Outstanding questions).

Viral factors that contribute to neuropathogenesis

There is currently limited understanding of viral factors that contribute to the neuropathogenesis of HPAI H5Nx viruses. Even though several studies identified viral factors that contribute to the replication efficiency in mammals, or transmission among them, these rarely investigate the contribution of these factors to neuropathogenesis (see Outstanding questions). However, it is known that the neuropathogenesis of HPAI H5Nx viruses cannot be attributed to a single viral protein.

Sialic acid binding preference

HPAI H5Nx viruses bind preferentially to α(2,3) SIA, but whether the recognition of specific SIA contributes to neuroinvasion and neurotropism is currently unknown. Viruses that recognize either α(2,3)- or α(2,6)-linked SIA attach to the olfactory mucosa of humans and ferrets. Furthermore, ferret studies have shown that both viruses that recognize α(2,3)- or α(2,6)-linked SIA are able to replicate within the olfactory mucosa, suggesting that SIA preference is not a major determinant in the ability to infect and replicate in cells of the olfactory mucosa [20,104]. Within the olfactory mucosa, highly polysialylated OSNs protrude through the submucosa into the environment where influenza A virus particles can directly attach and bind (Figure 1). However, which SIAs are specifically expressed on cilia of OSN is unknown [105]. The nerve endings of other CNs are mainly in contact with respiratory epithelial cells that express α(2,6) SIA and are therefore predominantly target cells for seasonal and pandemic influenza viruses [61,106]. With regard to the neurotropism, HPAI H5N1 virus [which binds α(2,3) SIA], attached and replicated more efficiently in neuron and astrocyte-like cell lines than viruses that recognized α(2,6)-linked SIA (pH1N1 and H3N2) [52]. These data suggest that the recognition of α(2,3)-linked SA might not play an important role in the neuroinvasive potential of HPAI H5Nx viruses, but that it might contribute to neurotropism.

Multibasic cleavage site

The HA proteins of HPAI viruses, including HPAI H5Nx viruses, contain a multibasic cleavage site (MBCS). The MBCS allows HA cleavage and activation by ubiquitously expressed proteases.

This is in contrast to the monobasic cleavage site of LPAI, seasonal and pandemic influenza viruses that are cleaved by specific trypsin-like serine proteases such as human airway trypsin-like protease (HAT), transmembrane serine protease 2 (TMPRSS2), TMPRSS4 or matriptases, which are not ubiquitously expressed [107–109]. The presence of the MBCS coincides with the systemic spread of HPAI viruses in birds, especially poultry species, and likely also in mammals [110]. In ferrets, the MBCS is critical for the neuroinvasion of HPAI H5N1 virus along the olfactory nerve. Deletion of the MBCS from H5N1/Indonesia/2005 virus prevented virus invasion into the CNS in ferrets, likely because replication within the olfactory mucosa was less efficient than H5N1/Indonesia/2005 with an MBCS [20]. However, the MBCS alone is not sufficient for neuroinvasion, as the insertion of a MBCS in a seasonal H3N2 virus did not increase the neuroinvasive potential in ferrets [111]. In mice and ferrets, virus spread to the CNS was not consistently observed after inoculation with different HPAI H7 viruses [112].

The presence of an MBCS in HPAI H5Nx viruses also contributes to the neurotropism. *In vitro* studies showed that H5N1/Indonesia/2005 both with and without the MBCS replicates in neuron-like cell lines and primary mouse cortex cells, although H5N1/Indonesia/2005 without the MBCS replicated less efficiently, despite replicating equally efficiently in MDCK cells [52]. Together, these studies indicate that the presence of MBCS contributes to the neuroinvasive and neurotropic potential of HPAI viruses, but the presence of a MBCS alone is not sufficient for neuroinvasion nor neurotropism.

Polymerase genes

The replication efficiency of influenza A viruses in mammalian cells is largely dependent on the viral polymerase genes PB1, PB2, and PA. A genetic screen has shown that mutations that increase polymerase activity in HPAI H5Nx viruses, like a single amino acid substitution in PB1 (N105S) or PB2 (E192K, Q591K, E627K, D701N or D701V), increase replication in the nasal turbinates, which correlated with higher virus titers in the brains of inoculated mice [113]. How these amino acid substitutions directly relate to the neuropathogenesis of HPAI H5Nx virus is not known. The E627K amino acid substitution in PB2 is one of the best studied in the light of mammalian adaptation, but in mice and ferrets, the E627K substitution also increases the neuroinvasive potential [54,68,114]. Furthermore, the acquisition of 627K, or other amino acid substitutions that increase polymerase activity (PB1-117G and 635T), within the CNS has been observed in naturally infected foxes and experimentally inoculated ferrets [30,60,80]. Together, these studies suggest that an increased polymerase activity might influence the neuroinvasive and neurotropic potential, possibly through more efficient replication.

Other factors contributing to neuropathogenesis

PB1-F2: Within the PB1 gene segment, an alternative start codon gives rise to the PB1-F2 protein. Amino acid position 66 in PB1-F2 was shown to be associated with increased neuroinvasion and neurotropism. In experimentally inoculated mice and ferrets, amino acid substitution N66S in PB1 increases the neuropathogenicity of HPAI H5N1 virus [115], most likely through suppression of the type I IFN response [116].

NS1: The nonstructural protein NS1 antagonizes the host innate immune responses through interacting with multiple host proteins [117]. One of these host proteins is cleavage and polyadenylation specificity factor 30 (CPSF30), a cellular factor required for processing of cellular pre-mRNA [118]. NS1 amino acid residues at position 103 and 106 are critical for a stronger interaction with CPSF30, which was associated with a faster spread of HPAI

H5N1 virus, especially in CNS cells [119]. *In vitro* evidence suggests that overexpression of NS1 derived from HPAI H5N1, but not pH1N1 virus, affects synaptic plasticity in rat neurons [120], most likely by interacting with PSD-95, a scaffold protein localized at the postsynaptic density [103].

Intervention strategies and treatment options

In view of the sensitivity of H5Nx viruses to neuraminidase inhibitors and the lack of approved vaccines, the CDC[iii] and the WHO[iv] recommend use of neuraminidase inhibitors. However, the efficacy of this approach in humans to prevent or treat neurological complications is poorly characterized. Hints on the efficacy of potential therapeutic intervention strategies, such as vaccination or antivirals, to prevent neurological disease can be found in preclinical studies. Mouse and ferret studies, for instance, have shown that vaccination with a homologous HPAI H5 vaccine prevented or reduced HPAI H5Nx virus neuroinvasion via the olfactory nerve [29]. Virus replication in the olfactory mucosa was less abundant or even absent in vaccinated ferrets, suggesting that vaccination limits virus spread to the CNS along the olfactory nerve [29]. Whether protective responses induced by the vaccine are effective, and how broad they are, is not well known and needs further investigation. Of note, a heterologous H3N2 vaccination did not prevent HPAI H5N1 virus neuroinvasion into the CNS in ferrets [25]. A comprehensive comparison of different vaccination strategies should reveal the optimal strategy to prevent CNS invasion by different HPAI H5Nx virus isolates. Combining virological, immunological, and pathological analyses in these studies could provide important insights into the correlates of protection.

Differences in antiviral effectiveness, dose regimens, and virus isolates were observed across various studies. In one ferret study, prophylactic oseltamivir did not prevent HPAI H5N1 virus replication in the olfactory mucosa, neuroinvasion, or virus spread throughout the CNS [29]. However, other studies revealed a reduction in virus titers or spread in the brain and neurological signs [121,122], and in mice, baloxavir marboxil did reduce virus titers in the CNS [123]. Future studies should reveal the potential of antivirals to prevent CNS invasion or reduce virus replication within the CNS, as well as efficient dosing regimens. These studies should assess the bioavailability of the different antivirals in different anatomical locations in the CNS, as, for example, oseltamivir, one of the most frequently used antivirals against influenza, has low bioavailability in the CNS [124,125].

Altogether, comprehensive studies should reveal the efficacy of vaccines and antivirals to prevent invasion and virus replication in the CNS (see Outstanding questions). As the majority of studies so far focus on the reduction of virus replication in the lungs, future studies should include analyses of the olfactory mucosa and different anatomical parts of the CNS. These studies have a broad impact in the context of pandemic preparedness and should complement the focus on treatment of HPAI H5Nx virus infections, as neurological disease has been associated with past influenza A virus pandemics.

Concluding remarks

The global spread of HPAI H5Nx viruses of the Gs/Gd lineage in wild birds is a unique event that affects not only birds but also mammals. Since 2021, sustained circulation of HPAI H5Nx viruses in wild bird populations has resulted in a tremendous increase of infected bird species as well as transmission events to mammalian species. Transmission to mammals, presumably after feeding on sick or dead birds, and the high frequency of neurological diseases in these mammals, raises concerns. In humans, HPAI H5Nx viruses have also been associated with neurological disease, but the exact risk for neurological disease after infection with currently

Outstanding questions

Many mammalian species infected with HPAI H5Nx viruses develop neurological disease, but it remains unknown how often infection results in neuroinvasion and neurological disease and whether this differs among mammalian species. What is the frequency of virus invasion into the CNS in different mammalian species, including humans?

Neurological disease observed in HPAI H5Nx virus-infected mammals is often severe or even fatal. This might only reflect 'the tip of the iceberg', and it is likely that less severe neurological disease occurs unnoticed in at least some mammalian species. What is the spectrum of neurological disease in different mammalian species?

Differences in neuropathogenesis are observed among different isolates of the HPAI H5Nx Gs/Gd lineage viruses. Under similar experimental conditions, some viruses cause neurological disease in mammals, whereas others do not. Which viral factors and phenotypic characteristics are necessary, or essential, for the neuroinvasive, neurotropic, and neurovirulent potential of HPAI H5Nx viruses?

HPAI H5Nx viruses show a broad neurotropism infecting different CNS cells, resulting in a proinflammatory response in the CNS. What cell types and which cell-intrinsic and cell-autonomous mechanisms contribute to CNS disease?

Individual studies have revealed that vaccination or antiviral therapies can potentially interfere with the neuroinvasion into and spread of HPAI H5Nx viruses through the CNS, but this has not been studied comprehensively. Can intervention strategies be used to prevent or treat neurological disease caused by HPAI H5Nx viruses?

circulating HPAI H5Nx viruses, and whether this is lower than other mammals, is not known (see Outstanding questions).

In mammals, the development of HPAI H5Nx virus-associated neurological disease can occur without any evidence of respiratory disease. For example, virus replication within the olfactory mucosa can result in neuroinvasion and spread throughout the CNS, without virus replication in other parts of the upper or lower respiratory tract. This comes with diagnostic challenges, as respiratory samples can test negative, despite efficient virus replication in the CNS. Therefore, it is important to increase awareness among veterinarians, health care workers, and neurologists to be vigilant about HPAI H5Nx viruses causing neurological disease, without the presence of overt respiratory disease [42].

The spectrum of CNS disease associated with HPAI H5Nx infection in different mammalian species remains unclear (Figure 3). It is likely that only those cases that develop severe disease from a HPAI H5Nx virus infection are documented with the current surveillance systems and that HPAI H5Nx virus infections that result in less severe disease are missed. A recent study in the Netherlands revealed that HPAI H5N1 virus infections were detected in carnivores without obvious neurological signs or morbidity, with serological evidence for infection in about 20% of the study population [126]. Similarly, limited human data suggest that only few cases of severe encephalitis occur, but milder neurological complications are frequently detected (Table 4). Although the disease pyramid for neurological disease is influenced by virus strain, virus dose, exposure route, and the species infected, it is important to acquire more insight into the diversity of CNS complications, as well as the frequency of these complications (Figure 3). Furthermore, it is currently unclear which host factors influence the neuropathogenesis, and thus contribute to differences among species. Finally, long-term effects of HPAI H5Nx virus infection need to be studied. It is likely that encephalitis will result in long-lasting neurological deficits, such as those observed after West Nile virus and severe acute respiratory syndrome coronavirus 2 infections [18,127].

A comprehensive understanding of the phenotypic and genotypic characteristics of HPAI H5Nx viruses that contribute to the development of neurological disease is urgently needed. Several virus-intrinsic features have been associated with the neuropathogenesis of influenza A viruses, such as the recognition of α(2,3)-linked SIAs, the presence of a MBCS, and an increased polymerase activity. However, none of these are solely responsible for the neuroinvasive, neurotropic, and neurovirulent potential of HPAI H5Nx viruses. *In vivo* and *in vitro* models are indispensable to identify viral factors that contribute to or are essential for the neuroinvasion, neurotropism, and neurovirulence. Scalable *in vitro* hiPSC-derived neural models are particularly useful tools to investigate virus–neural cell interactions in detail, as well as to characterize the neurotropic and neurovirulent potential of newly emerging H5Nx and other viruses in a human-related model system.

To conclude, HPAI H5Nx viruses are unique among influenza A viruses in their neuroinvasive potential, their efficient replication within the CNS, and their potential to cause severe CNS disease in mammals. With the continuous circulation of HPAI H5Nx viruses worldwide, mammals, including humans, are at risk of being infected. Although sustained transmission among mammals infected with HPAI H5Nx viruses is rare, it is critical to monitor the spread of this virus. A collaborative One Health approach is required to gain insights into the frequency and the full spectrum of neurological disease in humans and animals and to identify viral factors that contribute to the neuropathogenicity of HPAI H5Nx viruses. Finally, awareness should be raised among animal and human health care workers regarding neurological complications associated with HPAI H5Nx viruses and the potential of these neurological manifestations to occur in the absence of respiratory disease.

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Declaration of interests

The authors declare no competing interests.

Resources

[i]www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a(h5n1)-reported-to-who-2003-2023-24-april-2023

[ii]https://nextstrain.org/flu/avian/h5n1/ha

[iii]www.cdc.gov/flu/avianflu/novel-av-treatment-guidance.htm

[iv]https://apps.who.int/iris/bitstream/handle/10665/205388/B0634.pdf?sequence=1

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