MINIREVIEW

Transolfactory neuroinvasion by viruses threatens the human brain

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Summary. – Viral neuroinvasion via the olfactory system has been investigated in a variety of virus-animal models by scientists in many fields including virologists, pathologists, and neurologists. In humans, herpes simplex virus type 1 (HSV-1), human herpesvirus 6 (HHV-6), Borna disease virus, rabies virus, and influenza A virus have been shown to take the olfactory route for neuroinvasion based on forensic and post-mortem specimens. This article briefly summarizes the anatomy, physiology, and immunology of the olfactory system and presents a battery of neurovirulent viruses that may threaten the human brain by invading through this peripheral pathway, especially focusing on two of the most intensively studied viruses – HSV-1 and influenza A virus. Viruses may insidiously invade the olfactory neural network not only to precipitate encephalitis/encephalopathy but also to promote the development of neurodegenerative and demyelinating disorders. Substantial information obtained by analyzing human specimens is required to argue for or against this hypothesis.

Keywords: virus; olfactory neuron; human brain; encephalitis; neurodegenerative disorders

Contents:

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1. Introduction

To gain access to the central nervous system (CNS), neurotropic viruses exploit a variety of peripheral neural pathways as well as the hematogenous route (Kristensson, 2011). Neurons are highly polarized cells with structurally and functionally distinct processes known as dendrites and axons that can be separated by large distances. Axons have a uniform arrangement of microtubules with plus ends that are distal to the cell body (plus-end-out), whereas dendrites have equal numbers of plus- and minus-end-out microtubules. Kinesin and dynein serve as motor proteins for anterograde and retrograde transports, respectively, of macromolecules along microtubules. Infectious agents usurp this transport system to gain entry into the nervous

system. HSV is rapidly transported along microtubules in the anterograde direction from the soma to the axon terminus and retrogradely in the opposite direction. Viral capsid/tegument and glycoproteins synthesized in the soma may be separately transported in the anterograde direction down the axons. They likely accumulate in varicosities and growth cones, where capsids invaginate vesicles and acquire their envelope glycoproteins (Diefenbach et al., 2008).

2. The olfactory system

Olfactory mucosa lines the posterodorsal nasal cavity in terrestrial animals. The olfactory neuroepithelium consists of a limited number of cell types that are arranged in a roughly laminar pattern, with sustentacular cells in the most apical location, followed by olfactory receptor neurons (ORNs) and then basal cells (Mori et al., 2005a; Gizurarson, 2012). Sustentacular cells perform a number of glial-like neuroprotective functions, and they express high levels of cytochrome P450 isoforms and glutathione S-transferases (Rodriguez et al., 2008). Mature ORNs are bipolar sensory receptors with an apical dendrite ending in a knob covered by 12 or more cilia over its apical surface in the neuroepithelium. Basal cells are multipotent progenitors that give rise to ORNs throughout the life of the animal. Unmyelinated axons of ORNs leave the neuroepithelium, penetrate the cribriform plate, enter the olfactory bulb, and form synapses in the glomeruli with dendrites of second-order neurons, i.e., mitral/tufted cells. Mitral/tufted cells, in turn, project centrally to olfactory and limbic structures, including the anterior olfactory nucleus, olfactory tubercle, olfactory cortex, amygdala, and entorhinal cortex. On the other hand, the olfactory bulb has retrograde connections with the cholinergic diagonal band, serotonergic dorsal raphe, and noradrenergic locus coeruleus. Additionally, dopaminergic neurons in the ventral tegmental area project to the anterior olfactory nucleus. Once in the olfactory bulb, neurotropic viruses can target these brain structures in both anterograde and retrograde manners.

The olfactory system exhibits several unique properties that are not typical of other sensory systems. First, the receptor molecules of ORNs are directly exposed to the external environment so that they are able to respond to volatile chemical stimuli. Second, these neurons have the capacity for uptake and transsynaptic transport of exogenous substances to the CNS. Third, the olfactory system forms direct connections to the frontal cortex without thalamic relay, whereas other sensory pathways of the visual, auditory, and somatosensory modalities consistently pass through the thalamus. Finally, similar to other epithelial cells, but unlike other neurons, ORNs undergo apoptosis and regeneration as a part of their normal turnover process that continues throughout adult life and they have an average life span ranging from 30

to 120 days (Oboti et al., 2011). Furthermore, neuronal stem cells in the subventricular zone of the adult brain predominantly migrate into the olfactory bulb, likely protecting the olfactory bulb neuronal circuits from damage by infectious and toxic agents (Loseva et al., 2009). The physiological processes of uptake and transsynaptic transport of molecules from the nasal cavity to the olfactory bulb potentially pose great risks for the CNS, namely neurological damage induced by environmental factors including viruses.

In addition, axons of ORNs cross the cribriform plate through channels formed by olfactory ensheathing cells. These channels penetrate into the ventral portion of the olfactory bulb and may serve as a potential entry route for viruses into the brain. Viruses may also cause early meningoencephalitis by entering the cerebrospinal fluid (CSF) from infected olfactory ensheathing cells (Bodewes et al., 2011). This notion of alternative olfactory pathway for neuroinvasion by viruses is supported by an in vitro study that demonstrated human herpes virus 6 (HHV-6) replication in olfactory ensheathing cells (Harberts et al., 2011). Furthermore, the PR8 strain of influenza A virus (H1N1), a non-neurotropic virus, may also infect olfactory ensheathing cells as well as microglia/macrophages along the olfactory nerve fibers (Leyva-Grado et al., 2009). It should be noted that the olfactory mucosa is also innervated by fibers from the trigeminal nerve, autonomic fibers of the cervical ganglion, and the nervus terminalis (Vilensky, 2014). The olfactory nerve, however, connects the nasal cavity directly with the CNS and may therefore be a fast and easy shortcut for viruses to invade the CNS (van Riel et al., 2015).

3. Innate immunity in the olfactory system

Innate immunity plays a central role in CNS protection against a variety of neurotropic viruses (Carty et al., 2014). Within the olfactory bulb, interferon (IFN)-α/β-dependent antiviral mechanisms efficiently inhibit viral spread and serve as a barrier against intranasally invading neurotropic viruses (Kalinke et al., 2011). Toll-like receptor 3 (TLR3) expression was significantly increased in the olfactory bulb of mice after intranasal pretreatment with polyinosinic-polycytidylic acid, a TLR3 agonist, and it conferred protection against a lethal intranasal challenge with HSV-1 (Boivin et al., 2008). TLR3 activation by dsRNA (synthetic or virus-derived) triggers downstream signals that lead to the activation of the IFN-β promoter. Recently, it has been shown that local olfactory bulb infection with both DNA and RNA viruses activates long-distance signaling that upregulates IFN-stimulated gene expression in uninfected remote regions of the mouse brain (van den Pol et al., 2014). In the absence of the IFN-α/β receptor, the brain was more vulnerable to viral infection due to the impaired induction of IFN-stimulated genes.

In addition, Iba1-immunopositive microglia/macrophages, the principal resident innate immune cells in the CNS, reside in the murine olfactory neuroepithelium where they undergo dynamic morphological reactions upon intranasal influenza A virus infection (Mori *et al.*, 2002). OX42-immunopositive microglia/macrophages also appear in the rat olfactory neuroepithelium after bulbectomy and participate in phagocytic activities (Suzuki *et al.*, 1995). In addition, Iba1-/annexin A3-immunopositive microglia/macrophages reside in the olfactory nerve fascicles of adult rats and cats (Smithson and Kawaja, 2010). These peripheral microglia/macrophages may play a key role in TLR3 responses and downstream antiviral IFN signaling (Suh *et al.*, 2009). On the other hand, adaptive immune responses barely contribute to neuroprotection against viral attack in the olfactory neuroepithelium (Tan and Stevenson, 2014). Neuroepithelial HSV-1 infection failed to elicit significant virus-specific antibody responses in the nasal-associated lymphoid tissue. Thus, innate immunity, especially IFN-α/β-dependent mechanisms, serves as the first-line defense against invading intranasal viruses.

Viruses seek to subvert host immune responses. For example, distinct amino-acid motif in the polymerase of influenza A virus, PB1/PA, interacts with retinoic acid inducible gene 1 (RIG-1) that subsequently inhibits RIG-1-mediated IFN signaling (Liedmann *et al.*, 2014). Additionally, the expression of annexin V in influenza A virus particles inhibits IFN-γ antiviral immune responses (Berri *et al.*, 2014).

4. Transolfactory neuroinvasion by neurotropic viruses in humans

Accumulating evidence supports the notion that certain neurotropic viruses enter the human brain via an olfactory transmission mechanism, namely HSV-1, HSV-2, HHV-6, Borna disease virus, rabies virus, influenza A virus, measles virus, Hendra virus, Nipah virus, and poliovirus (Table 1). Among these viruses, HSV-1, HHV-6, Borna disease virus, rabies virus, and influenza A virus have been detected in the human olfactory tissue samples.

In experimental settings, the olfactory pathway can also provide a route of neuroinvasion by some human neurotropic arthropod-borne viruses (arboviruses) including La Cross virus, Japanese encephalitis virus, Murray Valley encephalitis virus, St. Louis encephalitis virus, West Nile virus, Chikungunya virus, and Venezuelan equine encephalitis virus (Table 1). Of note, La Crosse virus, Murray Valley encephalitis virus, St. Louis encephalitis virus, and Venezuelan equine encephalitis virus infect the olfactory neuroepithelium after intraperitoneal or footpad subcutaneous challenge, probably via the hematogenous route, which is likely due to the unmyelinated nature of ORNs and the absence of a neuroprotective architecture equivalent to the blood-brain barrier of the CNS

in the neuroepithelium (Charles *et al.*, 1995; McMinn *et al.*, 1996). This phenomenon is of particular interest because infectious virus particles could be released from infected ORNs into the nasal cavity, creating a potential source of droplet infection to other individuals.

The transmission of arboviruses without involvement of arthropod vectors is often termed *direct transmission*. This mode of viral transmission has been demonstrated in various experimental virus-animal models and may be common among wild and domestic animals (Kuno, 2001). The practical importance of such direct transmission of arboviruses has been recognized with respect to livestock-mediated as well as laboratory-acquired human infections. Arbovirus, especially present in the form of aerosols, could incidentally access the human brain through the olfactory route (Johnson and Mims, 1968). Thus, it is reasonable to assign a biosafety level to each arbovirus for protection of laboratory workers against accidental infections (Kuno, 2001).

5. The vomeronasal system as a route of neuroinvasion by neurotropic viruses

The vomeronasal organ, a second peripheral chemosensory module in the nasal cavity, has attracted increased scientific attention as a primary sensor for detecting pheromones in vertebrates (Ibarra-Soria *et al.*, 2014). This bilateral, cigar-shaped organ is encapsulated in a bony and/or cartilaginous capsule known as the vomer. It is located at the base of the nasal septum, anterior and ventral to the main olfactory neuroepithelium. The vomeronasal organ contains bipolar chemosensory neurons and its axonal nerve fascicles cross the cribriform plate to terminate in the accessory olfactory bulb. In mice, HSV-1 and HSV-2 enter the brain by infecting vomeronasal chemosensory neurons, in addition to the olfactory counterparts, following experimental intranasal inoculation (Mori *et al.*, 2005b,c, 2006). HSV-1 infection is further transmitted from the accessory olfactory bulb (first relay) to the medial amygdala (secondary relay), and then to the bed nucleus of the stria terminalis and the ventromedial hypothalamus (third relay). Although pheromone-mediated controls of human behaviors have been recorded, the presence of the functional vomeronasal organ in humans, however, still remains controversial (Gizurarson, 2012; Verhaeghe *et al.*, 2013). The neuroinvasion of neurotropic microbes via this route will mean a lot to non-human vertebrates, which could explain various abnormal behaviors of infected animals.

6. Herpes simplex virus (HSV)

Herpesvirus lineages have emerged by virtue of coevolution with their specific hosts, that is to say, *cospeciation*. The

Table 1. Neurotropic viruses that may invade the human brain through the olfactory pathway

Virus	Family	Subfamily	Genus	Notes	References
HSV-1/HSV-2	*Herpesviridae*	*Alphaherpesvirinae*	*Simplexvirus*	See details in text.	
HHV-6	*Herpesviridae*	*Betaherpesvirinae*	*Roseolovirus*	Frequently detected by PCR in the olfactory bulb/tract specimens of human autopsy cases.	Harberts *et al.*, 2011
Borna disease virus	*Bornaviridae*		*Bornavirus*	Infects ORNs and spreads intraaxonally to the limbic structures in rats[a].	Carbone *et al.*, 1987
				Infects ORNs and spreads to the brain in spontaneously and intranasally infected rats.	Morales *et al.*, 1988
				Spreads into the brain via the olfactory route during rat cohabitation experiments.	Sauder and Staeheli, 2003
				Detected by PCR in the olfactory bulb and related regions in human autopsy brain tissues.	Haga *et al.*, 1997
Rabies virus	*Rabdoviridae*		*Lyssavirus*	Infects ORNs and performs strict transsynaptic transmission in mice[a].	Lafay *et al.*, 1991
				Caused airborne rabies in a laboratory worker with neuroinvasion via the olfactory route.	Conomy *et al.*, 1977
Influenza A virus		*Orthomyxoviridae*	*Influenza virus A*	See details in text.	
Measles virus	*Paramyxoviridae*	*Paramyxovirinae*	*Morbillivirus*	Spreads via the olfactory pathway to the olfactory and limbic systems in hamsters[a].	Zlotnik and Grant, 1976
Hendra virus	*Paramyxoviridae*	*Paramyxovirinae*	*Henipavirus*	Infects ORNs and spreads to the olfactory/limbic systems in aged mice without viremia[a].	Dups *et al.*, 2012
Nipah virus	*Paramyxoviridae*	*Paramyxovirinae*	*Henipavirus*	Infects ORNs and spreads intraaxonally throughout the CNS in hamsters[a].	Munster *et al.*, 2012
La Crosse virus[b]	*Bunyaviridae*		*Bunyavirus*	Infects ORNs and spreads centripetally in mice after intraperitoneal injection.	Bennet *et al.*, 2008
Poliovirus	*Picornaviridae*		*Enterovirus*	Infects the olfactory bulb and spreads centripetally initiating paralysis in monkeys[a].	Sabin and Olitsky, 1938
				Takes the olfactory route for neuroinvasion in monkeys after droplet nuclei infection.	Faber *et al.*, 1944
				Infects the olfactory bulb and spreads centripetally in poliovirus receptor transgenic mice[a].	Crotty *et al.*, 2002
Japanese encephalitis virus[b]	*Flaviviridae*		*Flavivirus*	Infects the olfactory bulb and spreads to the olfactory tract/cortex in piglets[a].	Yamada *et al.*, 2009
Murray Valley encephalitis virus[b]	*Flaviviridae*		*Flavivirus*	Infects the olfactory bulb and spreads centripetally after footpad injection.	McMinn *et al.*, 1996
St. Louis encephalitis virus[b]	*Flaviviridae*		*Flavivirus*	Infects ORNs and spreads to the CNS in hamsters/mice after intraperitoneal injection.	Monath *et al.*, 1983
West Nile virus[b]	*Flaviviridae*		*Flavivirus*	Invades the brain via the olfactory route after viral aerosol exposure in mice.	Nir *et al.*, 1965
Chikungunya virus[b]	*Togaviridae*		*Alphavirus*	Invades the brain via the olfactory route in mice[a].	Powers and Logue, 2007
Venezuelan equine encephalitis virus[b]	*Togaviridae*		*Alphavirus*	Infects ORNs and spreads centripetally in mice after subcutaneous challenge.	Charles *et al.*, 1995

[a]After experimental intranasal infection. [b]Vector-borne virus in nature but can directly invade the human brain under unusual circumstances (see details in text).

alpha subfamily is estimated to have diverged from the beta and gamma subfamilies approximately 200–220 million years ago and the ancestors of HSV appeared 70–80 million years ago (Mori and Nishiyama, 2005). Thus, the virus-host relationship is fundamentally stable and the virus does not usually precipitate lethal diseases in humans. HSV-1 is transmitted through saliva and respiratory secretions among humans. Typically, HSV-1 asymptomatically infects the respiratory and oropharyngeal mucosa in children. Approximately 90% of adults are seropositive for HSV-1 and carry the viral genome in a latent form in peripheral ganglions. Reactivation, which may result in herpetic lesions in the skin and mucous membranes, takes place upon exposure to stressors or following immunosuppression.

7. Herpes simplex encephalitis (HSE)

HSE is a rare disease that is associated with a high mortality rate and serious neuropsychological and neurobehavioral sequelae. Patients remain afflicted with HSE for life even if they have been treated very early and have made a good recovery (Kennedy and Chaudhuri, 2002). HSE is usually caused by HSV-1; however, HSV-2 can also be responsible for HSE, especially in neonates and immunocompromised hosts.

The view that HSV-1 can invade the CNS through the olfactory route originally stems from the site-specificity of HSE; the propensity of the virus to localize to the fronto-temporal region of the brain and the limbic system (Johnson and Mims, 1968; Twomey *et al.*, 1979). The virus may also spread from the trigeminal ganglion to the temporal and frontal cortices by traveling along the tentorial branches of the trigeminal ganglion to the meninges that cover the frontal and temporal lobes (Davis and Johnson, 1979). However, there has been no direct evidence to support this assumption. Furthermore, because neurons in the trigeminal ganglion principally project to sensory nuclei in the brainstem, the trigeminal pathway can only account for infrequent atypical cases of HSE with brainstem involvement (Livorsi *et al.*, 2010).

A number of lines of evidence favor the olfactory route of neuroinvasion by HSV-1 in HSE patients. Postmortem immunohistochemical mapping analyses of 29 HSE subjects demonstrated the presence of HSV-1 antigens in the olfactory and limbic structures, but not in regions that would indicate invasion via the trigeminal pathway (Esiri, 1982). Furthermore, HSV-related histopathological alterations were detected in the olfactory neuroepithelium, olfactory nerve, and olfactory bulb, but not in the trigeminal ganglion (Twomey *et al.*, 1979; Dinn, 1980; Ojeda *et al.*, 1983). Additionally, life-long olfactory dysfunction has been described in patients who survived HSE, thus highlighting viral damage to the olfactory neural network in the human brain (Landis *et al.*, 2010).

Animal experiments using HSV-1 largely recapitulated the olfactory system damage observed in humans. Following intranasal inoculation with HSV-1 +GC strain, rats contracted acute encephalitis involving regions of the olfactory and limbic systems, including the olfactory bulb, olfactory cortex, amygdala, hippocampus, and entorhinal cortex, and they demonstrated spatial memory deficits (Beers *et al.*, 1993, 1995). Moreover, a mouse model of HSE using the HSV-1 strain 17 syn+ revealed similar neuropathological and neurobehavioral abnormalities (Armien *et al.*, 2010). Following intranasal infection with the HSV-1 strain SC16, mice developed an asymptomatic and transitory CNS infection that was characterized by viral attack of the olfactory bulb, amygdala, and hippocampus, as well as of the olfactory, cingulate, fron-

tal, temporal, and entorhinal cortices (Boggian *et al.*, 2000). The HSV-1 strain H129 targets brain structures within the projections of the olfactory pathway including olfactory and entorhinal cortices upon intranasal inoculation (Hudson *et al.*, 1991). It is important to note that in another study, the SC16 virus targeted the murine olfactory neuroepithelium but rarely reached the olfactory bulb, which was likely due to differences in the animal strains and ages of the mice between the studies (Shivkumar *et al.*, 2013).

8. Role of HSV accessory genes in the olfactory neuroinvasion

HSV accessory genes are defined as non-essential genes that are not required for replication in cultured cells but play key roles in virus-host interactions. These genes may help neurovirulent viruses invade the brain through the olfactory route (Mori, 2012). Wild type HSV-2 strain 186 infects a fraction of ORNs without inducing neuronal death and it is transmitted to the CNS where it initiates lethal encephalitis. In sharp contrast, the *US3*-disrupted mutant strain, L1BR1, immediately triggers neuronal apoptosis in peripheral neurons upon infection, thus blocking viral transmission to the brain (Mori *et al.*, 2006). The *US3*-repaired mutant strain, L1B⁻11, behaves similarly to the wild-type virus. These observations suggest that viral US3 protein kinase inhibits virus-induced apoptosis of ORNs, thus enabling efficient viral transmission into the CNS. HSV-1 *US3* also plays a role in resistance to IFN. The growth of *US3*-disrupted virus is significantly suppressed in IFN-treated HEp-2 cells when compared to wild-type and *US3*-reconstituted virus (Piroozmand *et al.*, 2004). A prominent increase in intracellular expression of TLR3 and IFN-inducible myxovirus resistance A protein have been recorded in *US3*-deficient HSV-1-infected U937 human monocytic cells, but not in cells infected with corresponding parental and *US3*-rescued viruses (Peri *et al.*, 2008). Furthermore, HSV-1 US3 protein kinase cooperates with glycoprotein B to rapidly inhibit CD1d antigen presentation and natural killer T-cell activation during the initial antiviral response (Rao *et al.*, 2011). Additionally, ICP0 impairs the activation of IFN regulatory mediators such as IFN-γ-inducible protein 16 (Lanfranca *et al.*, 2014). Virion host shutoff protein counteracts the antiviral action of IFN-inducible viperin protein (Shen *et al.*, 2014). ICP34.5 confers neurovirulence upon HSV by regulating IFN-α/β responses in mice (Davis *et al.*, 2014). Thus, HSV has developed a variety of mechanisms to limit hosts' antiviral responses.

The HSV-1 strain HF10 lacks a functional *UL56* and exhibits impaired neuroinvasiveness via the olfactory route in mice (Mori *et al.*, 2005c). UL56 is a C-terminal-anchored type II membrane protein that is expected to be inserted

into the viral envelope. Interestingly, UL56 protein physically associates with kinesin motor protein KIF1A, and it is most likely involved in anterograde axonal transport of the viral envelope loaded with viral glycoproteins along axons (Koshizuka *et al.*, 2005).

9. HSV latency and persistency in the central nervous system

Viruses may silently invade and establish a latent/persistent infection in the olfactory bulb and a particular set of limbic structures. PCR detected HSV-1 DNA in 15.5% of the olfactory bulbs collected from 97 individuals during forensic postmortem analyses (Liedke *et al.*, 1993). PCR detection of HSV-1 genome in 17.5% of the olfactory bulbs and less frequently in the amygdala and hippocampus collected from 40 patients dying of non-neurological causes provided evidence in support of olfactory neuroinvasion during the course of a neurologically silent infection (Baringer and Pisani, 1994). Asymptomatic HSV-1 persistence in the olfactory epithelium and bulb was recapitulated using murine models (Drummond *et al.*, 1994; Boggian *et al.*, 2000). It should be noted that HSV infection of the CNS through the olfactory pathway, even if it takes place silently, may bring about learning and behavioral deficiencies in children and adults (Becker, 1995). These findings lead to an assumption that HSV reactivation may originate in the olfactory as well as in limbic structures. Of interest, the olfactory neuron-specific transcription factor, Olf-1, activates the promoter of *RL2* and drives the expression of ICP0, which may in turn facilitate productive infection and reactivation within the olfactory system (Devireddy and Jones, 2000). Thus, it is conceivable that HSE in children occurs during the course of primary HSV-1 infection, whereas in adults HSE arises from the reactivation of the virus in the olfactory and/or limbic structures.

On one hand, the recent improvements in sanitizing environments have increased the age of primary HSV-1 infections, and it may bring about new health problems in the near future. On the other hand, HSV-1 frequently establishes continuous and productive infections in the frontal and temporal cortices of aged individuals through expression of HSV-1 accessory genes. This has been implicated, at least in part, in the pathogenesis of Alzheimer's disease (Mori, 2010; Itzhaki, 2014).

10. Influenza A virus

Influenza A virus primarily infects the respiratory system in humans and sporadically induces extra-respiratory system complications including influenza encephalitis/encephalopathy. Influenza A virus neuroinvasion along the olfactory pathway has been extensively investigated using animal models, including mice and ferrets. The recombinant influenza A virus strain, 90/Ho1, which has the RNA segment 2 derived from A/Hong Kong/1/68 (H3N2) and other segments from A/fowl plague/Rostock/34 (H7N1), entered the brain of neonatal mice through the olfactory route following intranasal challenge (Reinacher *et al.*, 1983). This can take place even in the presence of neutralizing antibodies in their serum, thus ruling out the hematogenous spread of the virus. The A/WSN/1933 (H1N1) virus, which is closely related to the 1918/1919 influenza pandemic, invades the olfactory bulb and several interconnected brain structures in mice (Aronsson *et al.*, 2003). The virus can cause transient infection in the CNS, but leads to persistent changes in emotional and cognitive functions as well as elevated transcriptional activity of genes encoding synaptic regulatory proteins in the amygdala and hypothalamus (Kristensson, 2006). By contrast, infection by the non-neurotropic PR8 strain (H1N1) of influenza A virus was restricted to microglia/macrophages in the olfactory nerve fascicle and bulb, but induced widespread CNS production of proinflammatory cytokines including interleukin 1β and tumor necrosis factor-α (Leyva-Grado *et al.*, 2009) and influenced the acute phase response including hypothermia (Leyva-Gardo *et al.*, 2010; Zielinski *et al.*, 2013).

We have previously shown that the recombinant influenza A virus strain R404BP, which possesses the neuraminidase and matrix genes from the WSN strain and the rest from the non-neurovirulent A/Aichi/2/68 strain (H3N2), induced apoptosis in ORNs upon infection following intranasal challenge in mice (Mori *et al.*, 2002). Virus-infected ORNs and sustentacular cells in the vicinity upregulated the expression of Fas ligand and activated the c-Jun N-terminal kinase signal transduction pathway. Apoptotic bodies were totally cleared by activated Iba1-immunopositive microglia/macrophages in the olfactory neuroepithelium. Viral components including nucleic acids and proteins remained undetectable in the olfactory bulb. All of the mice survived the infection and failed to exhibit any clinical symptoms. An introduction of one plaque-forming unit of virus directly into the olfactory bulb was sufficient to kill 100% of the mice. Taken together these findings suggested that virus-induced neuronal apoptosis in ORNs is a protective host response that hinders virus transmission into the brain (Mori *et al.*, 2004). This phenomena, however, appears to be atypical, since many reports documented viral infections of ORNs in the absence of neuronal death in the neuroepithelium.

11. Highly pathogenic avian influenza

Recent studies have largely focused on highly pathogenic avian influenza (HPAI) H5N1 viruses that bring about a high

incidence of neurological complications in many animal species and sporadically in humans (Korteweg and Gu, 2008). During the 1997 Hong Kong incident, the avian H5N1 strains, HK156 and HK483, were isolated from the throat aspirates of a three-year-old boy who was the index case and subsequently developed Reye's syndrome and a 13-year-old girl who was the third case, respectively. Thus far, H5N1 virus has been detected in the CNS of a limited number of human cases.

In mouse models, HK483 virus takes the olfactory route for neuroinvasion, although other pathways through the trigeminal, vagal, and sympathetic systems are also possible (Park *et al.*, 2002; Tanaka *et al.*, 2003; Iwasaki *et al.*, 2004). Viremia may not largely contribute to the viral dissemination to the brain. In addition, HPAI A/Vietnam/1203/04 (H5N1) virus invades the murine brain, at least in part, through the olfactory route after snout infection, inducing neuroinflammation and neurodegeneration (Jang *et al.* 2009). In this experimental system, the virus attacked various brain regions. Of note, phosphorylated alpha-synuclein (pSer129SYN) is detectable in neurons present in infected areas and aggregated alpha-synuclein, a hallmark of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases, in the hippocampus, cortex, and brainstem. In addition, a significant number of dopaminergic neurons in the substantia pars compacta gradually die through a mechanism involving apoptosis, reminiscent of the postencephalitic Parkinsonism that could be associated with the 1918/1919 influenza pandemic (Gamboa, 1974; Kristensson, 2006).

Many H5N1 strains isolated from various Asian countries infected the olfactory neuroepithelium and bulb at early stages in ferrets (Bodewes *et al.*, 2011; Plourde *et al.*, 2012; Schrauwen *et al.*, 2012; Yamada *et al.*, 2012; van Riel *et al.*, 2015). In ferret nose tissues, abundant attachment of the H5N1 virus to the apical side of the olfactory neuroepithelium was noted, whereas no attachment of the virus to the apical side of the respiratory epithelium was detected (Schrauwen *et al.*, 2012). The multibasic cleavage site in the hamagglutinin of the H5N1 virus plays a critical role in the olfactory spread of the virus (Schrauwen *et al.*, 2012). The virus first appears in mitral cells in the olfactory bulb and spreads centripetally to interconnected structures of the brain. Three-dimensional analysis of viral distribution in the ferret brain unequivocally identified the olfactory system as a major route of viral neuroinvasion (Shinya *et al.*, 2011). The H5N1 virus presented more extensive CNS spread than pandemic H1N1 virus, whereas seasonal H3N2 virus exhibited minimum neurotropism in ferrets (van den Brand *et al.*, 2012). A novel HPAI virus (H7N9) invades the olfactory bulb following intranasal infection in ferrets (Xu *et al.* 2013, 2014). It has been demonstrated that the H7N9 virus harbors the nucleoprotein that subverts the antiviral action of human myxovirus resistance A protein (Riegger *et al.*, 2015).

12. Influenza A virus in human olfactory nervous tissues

The first evidence of influenza virus entry through the olfactory route in a human was recently described, with viral antigens being detected in the olfactory bulb and tract of postmortem brain samples (van Riel *et al.*, 2014). The patient was an 11-month-old girl with a severe immunodeficiency, the detail of which remains unknown. Sequence analysis of RNA extracted from the bronchoalveolar lavage revealed the presence of a seasonal influenza A H3N2 virus. In addition, three strains of influenza A virus, i.e., seasonal H3N2, pandemic H1N1, and H5N1, have been demonstrated to attach to the apical side of human and ferret olfactory mucosa, strengthening the likelihood of the olfactory route of neuroinvasion in humans (van Riel *et al.*, 2014). Of special significance is the fact that two mutations (L226Q and S228G) in the hemagglutinin gene of A/Hong Kong/1/68 (H3N2) dramatically augmented the viral neurovirulence for the olfactory mucosa (Van Poucke *et al.*, 2013). Thus, CNS invasion of seasonal H3N2 virus could be explained not only by immune defects of the host but also by the increased neurotropism of the virus, precipitated by a few mutations in the viral genome.

Furthermore, indirect evidence supports influenza A virus entry into the human brain via the olfactory route. H3N2 virus RNA could frequently be detected in the CSF of children with influenza-associated acute encephalitis/encephalopathy at early stages of infection (Fujimoto *et al.*, 1998). A previously healthy 26-year-old man developed a fatal encephalopathy without any respiratory disease. Real-time PCR detected influenza A (H1N1) pdm09 virus in a brain biopsy specimen but did not reveal the presence of the viral RNA in the lung (Simon *et al.*, 2013). In addition, H5N1 virus was detected in the CSF of the four-year-old boy who died of acute encephalitis, without any respiratory disease at the moment of hospitalization (de Jong *et al.*, 2005).

13. Concluding remarks

Experimentally, a wide variety of neurotropic viruses make use of the olfactory route for neuroinvasion into the CNS. In humans, however, there is a scantiness of direct evidence demonstrating this route of CNS entry because of the restricted availability of human nervous tissues from the nasal epithelium to the olfactory bulb/tract. Detailed investigation of such human specimens will expand our understanding of the neuropathogenesis of virus-induced encephalitis/encephalopathy and some neurodegenerative disorders in which early olfactory dysfunction and olfactory bulb pathology have much been described, such as Alzheimer's and Parkinson's diseases (Doorn *et al.*, 2014). Furthermore, early and prominent olfactory dysfunction and

pathology are common in demyelinating disorders including multiple sclerosis (DeLuca *et al.*, 2015). Common respiratory viruses may be involved in the pathogenesis of these diseases (Majde, 2010). According to the *hit-and-run* theory, although viruses are eventually eradicated by the immune system at early stages of the infection, they have a potential to trigger CNS disorders by inducing severe neuroinflammation from the olfactory network (Leyva-Grado *et al.*, 2009).

Finally, intranasal delivery of drugs, nucleic acids, and cells through the olfactory conduit to the CNS will drastically improve therapeutic and prophylactic approaches to neurological and neuropsychiatric diseases in the future (Dhuria *et al.*, 2010; Kanazawa T *et al.*, 2013; Danielyan *et al.*, 2014).

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