H1N1 influenza virus induces narcolepsy-like sleep disruption and targets sleep–wake regulatory neurons in mice

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An increased incidence in the sleep-disorder narcolepsy has been associated with the 2009–2010 pandemic of H1N1 influenza virus in China and with mass vaccination campaigns against influenza during the pandemic in Finland and Sweden. Pathogenetic mechanisms of narcolepsy have so far mainly focused on autoimmunity. We here tested an alternative working hypothesis involving a direct role of influenza virus infection in the pathogenesis of narcolepsy in susceptible subjects. We show that infection with H1N1 influenza virus in mice that lack B and T cells (Recombinant activating gene 1-deficient mice) can lead to narcoleptic-like sleep–wake fragmentation and sleep structure alterations. Interestingly, the infection targeted brainstem and hypothalamic neurons, including orexin/hypocretin-producing neurons that regulate sleep–wake stability and are affected in narcolepsy. Because changes occurred in the absence of adaptive autoimmune responses, the findings show that brain infections with H1N1 virus have the potential to cause per se narcoleptic-like sleep disruption.

Influenza A virus | lateral hypothalamus | orexin | locus coeruleus | noradrenaline

Narcolepsy is a rare, lifelong sleep disorder associated with loss of neurons expressing the neuropeptide orexin/hypocretin (Orex/Hcrt), which reside in the lateral hypothalamic area (LH) (1, 2). This disorder is characterized by many symptoms of disturbed sleep, dominated by sleep–wake instability and fragmentation and, in narcolepsy type 1, by cataplexy (3–5). Narcolepsy is strongly associated with the HLA DQB1*06:02 haplotype, and more weakly with polymorphisms in the genes encoding TNF-α and TNF receptor II (6), as well as the T-cell receptor-α chain (7) and P2RY11 (8). The genes encoding the LH histocompatibility system in humans are located in the MHC region, which is associated with more diseases, mainly of infectious or autoimmune nature, than any other region of the genome (9). Because narcolepsy is primarily related to a single allele, the class II HLA-DQB1*06:02 haplotype, an autoimmune mechanism, has been hypothesized for the loss of Orex/Hcrt neurons, although the antigens involved have not been identified (3–6). In light of the high rate of discordance of narcolepsy among monozygotic twins, critical roles of environmental triggering factors have also been proposed (5, 10). Additional risk alleles in both MHC class I and II have also been identified, which might influence adaptive immune response and virus clearance. The association of HLA alleles with narcolepsy is, thus, more complex than hitherto presumed (11, 12).

Although the etiology of narcolepsy remains to be understood, an increased narcolepsy incidence in northern and western European countries since 2009 is being currently discussed (13).

On the one hand, association with one of the influenza vaccines administered during the 2009–2010 influenza A H1N1 virus pandemic has been suggested (14, 15). On the other hand, influenza virus infections have previously been reported to represent risk factors for narcolepsy (16) and seasonal onset of this disorder did indeed increase following the 2009–2010 pandemic in China, where there was no concurrent vaccination campaign (17). In Europe, relatively few patients with narcolepsy reported influenza-like symptoms preceding the illness (18). However, serological studies have suggested a high rate of mild or asymptomatic infections during the 2009 pandemic (19). The lack of laboratory verification of previous influenza exposure in most reported cases of narcolepsy precludes conclusions on a causative role for influenza virus in narcolepsy.

Influenza virus infections have, however, been associated with a number of functional disturbances in the nervous system. These could be secondary to respiratory tract infections. For example, daytime somnolence is common as part of sickness behavior caused by the release of proinflammatory cytokines, such as IL-1β and TNF-α (20). An exaggerated cytokine release,
“cytokine storm” (21), may also be involved in the pathogenesis of the bilateral thalamic necrosis reported in Japanese children suffering from influenza infections (22).

Certain strains of influenza A virus, especially avian strains, can also cause primary lesions by invading the nervous system (23). After experimental intranasal instillation, such avian strains can spread by axonal transport to the brain along both the olfactory and trigeminal nerve pathways (24).

In this context, the aim of the present study was to determine whether H1N1 influenza A virus per se has the potential to cause changes in the sleep pattern and to target neurons of the sleep–wake-regulatory network (25–27). We here used mice with a targeted deletion of the Recombinant activating gene 1 (Rag1<sup>−/−</sup>), lacking B and T cells (28), and localized intranasal instillation of a mouse-neuroadapted H1N1 strain of influenza A virus, WSN/33. These animals cannot mount an MHC-dependent adaptive immune response that could clear the infection, and do not exhibit lower respiratory tract infections upon the virus exposure (29), thus allowing studies on primary effects of the infection on the brain. Rag1<sup>−/−</sup> mice therefore provide a tool to disclose viral targets in individuals with an inefficient viral clearance, a condition recently suggested in narcoleptic patients (12).

**Results**

**Sleep–Wake Changes.** Mice were instilled intranasally with H1N1 influenza A virus or saline. From the third to fourth week postinfection onwards, some infected mice started to show reduced gain in weight. For ethical reasons, all mice were killed before reaching 15% weight loss (Fig. 1). Until then, the animals did not show any visible sign of sickness behavior.

Wake and sleep states were recorded before (baseline) and during the infection using electroencephalography (EEG), accelerometric recording of body movements, as well as electromyography (EMG). Sleep includes two sleep states, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and normal sleep consists of cycles of SWS epochs followed by REM sleep episodes. Sleep and wake, characterized by behavioral quiescence or activity, respectively, are defined by distinct neurophysiological parameters (Fig. 2A). In particular, REM sleep is characterized by EEG traces similar to wake but with loss of skeletal muscle tone (Fig. 2A and SI Appendix, Fig. S1).

Narcolepsy is characterized in humans by sleep–wake fragmentation, with daytime sleep episodes and nocturnal awakenings as well as sleep structure alterations (5). These include instability of REM sleep regulation, with the frequent occurrence of so-called sleep-onset REM sleep (SOREM) episodes, in which the onset of REM sleep is preceded by wake instead of SWS (30). These alterations (Fig. 2A and SI Appendix, Fig. S1) have also been reported in murine models of narcolepsy (31, 32).

Sleep and wake states were analyzed in infected Rag1<sup>−/−</sup> mice at 2, 3, and 4 wk postinfection (in the fourth week, EEG was analyzed 2–6 d before killing, when an initial loss of weight was seen in only two of the six EEG-recorded mice) and compared with baseline recordings and with matched, saline-treated, control Rag1<sup>−/−</sup> mice. Notably, in the control Rag1<sup>−/−</sup> mice no significant changes were detected between the baseline EEG recordings and those obtained at 2, 3, and 4 wk after saline treatment. This finding shows that the EEG recording implant and procedures did not cause sleep–wake changes over time per se (SI Appendix, Fig. S2). Several changes in the sleep–wake pattern were instead detected over time in the infected Rag1<sup>−/−</sup> mice.

During the light phase, when nocturnal rodents sleep most of the time (as shown in the control hypnograms in Fig. 2B and SI Appendix, Fig. S3A), no significant changes in the total time spent in each state (wake, SWS, REM sleep) were recorded in the infected Rag1<sup>−/−</sup> mice in the second, third (SI Appendix, Fig. S3C), and fourth weeks postinfection (Fig. 2C). No significant alterations of the analyzed sleep and wake parameters were found in the infected mice in the second and third weeks postinfection (SI Appendix, Fig. S3 D–F), whereas in the fourth week postinfection the sleep–wake pattern became altered. In particular, the number of state episodes (Fig. 2C) and of sleep–wake transitions was increased, and the time spent in each state episode was decreased (Fig. 2C), with reduced mean REM sleep latency (Fig. 2C). This resulted in a marked fragmentation of sleep in the fourth week postinfection, with rapid cycling between state episodes during the light phase (Fig. 2B). In addition, SOREM episodes (Fig. 2A and SI Appendix, Fig. S1), not seen in the saline-treated controls, appeared in the fourth week postinfection (Fig. 2B). Video-recording was not possible because of requirements of the facilities for infected animals, but behavioral immobility during SOREM episodes was confirmed in the infected mice by accelerometric recording (SI Appendix, Fig. S1).

During the dark phase, when nocturnal rodents are mostly awake (Fig. 2B and SI Appendix, Fig. S3B), no significant changes were found in the second and third weeks postinfection (SI Appendix, Fig. S3 G–J). During the fourth week postinfection, the total time spent in wakefulness was decreased and that spent in REM sleep was increased (Fig. 2C). In addition, the number of state episodes (Fig. 2C) and sleep–wake transitions was markedly increased in the infected mice, in which the mean duration of wake episodes decreased (Fig. 2C). This resulted in the frequent intrusion of sleep episodes into wakefulness in the infected mice (Fig. 2B). Furthermore, SOREM episodes occurred among the infected mice also during the dark phase (Fig. 2B).

Importantly, the percentage of body weight changes was not significantly correlated with the total number of sleep–wake transitions [r = −0.551, P (two tailed) > 0.05] observed in the infected group during the fourth week postinfection.

**Spectral Power Analysis.** No significant changes in the spectral power of the EEG traces were found over time for any of the sleep–wake phases in the saline-exposed control group. No differences in the spectral power of any bandwidth for wakefulness, SWS, and REM sleep were seen in the infected mice compared with saline-treated controls. In the infected mice a significant increase in the spectral power of the SWS 0-band was instead observed in the fourth week postinfection during both the light and dark phases (SI Appendix, Fig. S4). These findings are similar to those observed in a conditional ablation model of Orex/Hcrt deficiency (33).
Innate-Immune Responses. Mediators of the innate-immune response are released by the host during the infection to limit viral spread. These mediators can also affect sleep and wakefulness (20). During the progression of the infection (17–39 d postinfection), brains from the infected animals harbored significantly increased levels of transcripts encoding TNF-α, IL-1β, and IFN-β, but not of that encoding inducible nitric oxide synthase, which is involved in SWS recovery after sleep deprivation (34) (SI Appendix, Fig. S5).

Influenza Virus Targets Brain Areas Involved in Sleep–Wake Regulation. Cells targeted by the virus were investigated by immunohistochemistry. Importantly, viral antigens were not detected in the lung at 28–29 d postinfection (SI Appendix, Fig. S6 A and B), indicating that the effect on vigilance states was not secondary to a pulmonary infection.

Highly neurovirulent avian strains of influenza A virus can invade the brain in ferrets and mice along the trigeminal and olfactory pathways following intranasal instillation (24). In line with this, viral antigens were seen in a few trigeminal ganglia neurons and were highly expressed in the dorsal trigeminal root central to the periphery-brain border (SI Appendix, Fig. S6 C–E). In the olfactory bulb (OB), the virus spread from the olfactory nerve layer to the deeper layers as revealed at early and late stages (Fig. 3). Notably, the infection was restricted to areas connected to the olfactory and trigeminal systems and there was no further spread into the neocortex or hippocampus, except in rare instances.

Overall the viral invasion displayed a distinct pattern in the brain, although with a considerable interindividual variability in viral antigen load also in the OB (Fig. 3B). The rostro-caudal spread was also mostly bilateral, with exception of the LH,
where, however, interindividual variations in viral load seemed to depend on the degree of infection of the OBs (Figs. 3C and 4 and SI Appendix, Fig. S11).

Caudal to the OB many, often intensely labeled cell groups encompassed both neurons and glia (Fig. 4 and SI Appendix, Fig. S6 F–I). These were located, inter alia, in the nucleus of the horizontal and vertical limbs of the diagonal band, piriform cortex, cortical amygdaloid area, LH, ventral tuberomammary nucleus (TMN), lateral mammillary nucleus, posterior hypothalamic nucleus, ventral tegmental area (VTA), dorsal and median raphe nuclei (DRN/MRN), locus coeruleus (LC), and surrounding nuclei, as well as trigeminal and facial nerve territories along the ventro-lateral aspects of the pons and medulla (Fig. 4). Viral antigens were not detected in the ventro-lateral preoptic nucleus or the suprachiasmatic nucleus.

We next investigated the chemical phenotype of the neurons targeted by the virus. Combinations of double-labeling showed that both Orx/Hcrt neurons and neurons containing melanin-concentrating hormone (MCH) were infected (Fig. 5A–D). This finding is of particular interest in view of the well-established involvement of both these peptidergic cell types in sleep–wake regulation (35). There was a reduction in numbers of Orx/Hcrt neurons (56 ± 19% loss) (Fig. 5A) in the LH of the infected side, whereas the MCH-immunolabeled neurons appeared preserved (3 ± 8% loss) (Fig. 5C). This is probably because the Orx/Hcrt innervation mainly targets the glomerular layer (GL) and granule cell layer (GCL) of the OB (SI Appendix, Fig. S7 A–E), whereas the MCH mainly projects to the GCL (SI Appendix, Fig. S7 F–J). Cresyl violet-stained adjacent sections revealed cell loss only in the heavily infected area (mainly Orx/Hcrt neurons) but not in the marginal zone (mainly MCH neurons), whereas contralateral, noninfected neurons appeared normal (Fig. 5 E–G). Triple-labeling by coinoculation with two additional antibodies, one against glutamic acid decarboxylase 67 (GAD67) to reveal GABAergic elements and one against the vesicular glutamate transporter type 2 (VGLUT2) to identify glutamatergic elements, indicated that the staining of nerve endings in the neuropil was well preserved on both sides (Fig. 5H). Moreover, microglia gradually increased from the periphery to the heavily infected center of the lesions, exhibiting a series of phenotypic changes suggesting activation (Fig. 5I and SI Appendix, Fig. S8 A–C). Astrocytes also showed features of activation in the periphery of the heavily infected area, in particular (Fig. 5J and SI Appendix, Fig. S8 E–F). Viral antigens were seen within astrocytes, but rarely in microglia (SI Appendix, Fig. S8 D and E).

Similar infection and double-staining patterns could also be detected in many other nuclei/areas involved in sleep–wakefulness regulation: (i) cholinergic ventral forebrain neurons (Fig. 6 A and B); (ii) histaminergic neurons in the TMN (Fig. 6 C and D); (iii) the transition zone between the VTA and the most medial aspects of the substantia nigra, overlapping with dopamine

Fig. 3. Viral antigen distribution in the OB and the LH. (A) At an early stage (14 d postinfection) after nasal infection, viral antigens are detected in in the olfactory nerve layer (ON; green color in adjacent Cresyl violet-stained section). (Scale bars: 200 μm; Inset is 20 μm.) At a late stage (28 d postinfection), viral antigens extensively label neurons and glia in all layers except the granular layer (GrO), as shown at three different levels (adjacent sections stained with Cresyl violet). Right sagittal drawing shows the viral spread. Pink color reflects severity of infection, and the dashed green arrow indicates the spreading pathway. (Scale bars, 1 mm.) (B) Overview of the mice analyzed for occurrence of viral antigens in the OB and the LH. Viral antigens are present in the LH of all mice with abundant OB infection as well as in one mouse with spread of virus into the ventricles, but not in the mice showing no or only a few infected cells in the OB (SI Appendix, Fig. S9) (mouse #6). (C) Summary of virus-infected area in the LH. The dashed lines in the Insert show the size of the infected area for six mice. Mouse #1 and #6 were subjected to EEG recording. Mouse #1 and #2 showed a clear loss of Orx/Hcrt neurons. 3V, 3rd ventricle; EPl, external plexiform layer; f, fornix; Gl, glomerular layer; IPl, internal plexiform layer; Mi, mitral cell layer; opt, optic tract.
tyrosine hydroxylase-positive) neurons (Fig. 6E and F); (iv) DRN/MRN neurons overlapping with serotonergic neurons (Fig. 6G and H); and (v) LC and adjacent areas overlapping with noradrenergic neurons (Fig. 6I). Activated astrocytes and microglia were abundant in these areas (SI Appendix, Fig. S9).

In the LC, degeneration of noradrenergic neurons occurred in the heavily infected area, surrounded by morphologically preserved neurons (Fig. 6I and J). Similarly to the LH, cell loss was seen in Creyl violet counter-stained adjacent sections (Fig. 6K–N). GABAergic and glutamatergic nerve endings were preserved also in the LC, even within the heavily infected area (Fig. 6O and P). Notably, the viral infection extended in most mice outside the LC “core” into the pontine reticular nuclei (oral, PnO), locus coeruleus (LC), and surrounding nuclei: for example, the lateral dorsal tegmental nucleus (LDtg), mesencephalic trigeminal nucleus (Me5). The overlying ependymal layer is also infected. (N–S) Caudally the infection essentially forms two bilateral columns along the ventro-lateral aspects of pons and medulla, associated with the trigeminal (5N) and facial nerve (7N) territories, extending into adjacent nuclei, such as the intermediate (IRt) and parvocellular nuclei at the pontine and medullary levels. (T) In addition, the nucleus raphe pallidus (RPa) and area postrema (AP) are labeled. ac, anterior commissure; Aq, aqueduct; CPu, caudate-putamen; LR4V, lateral recess of the fourth ventricle; LV, lateral ventricle; m5, motor root of the trigeminal nerve; MeA, medial amygdaloid nucleus; PAG, periaqueductal gray. (Scale bar, 2 mm.)

In one mouse, viral spread into the ventricles occurred, probably originating from infection in the OB extending into the olfactory part of the ventricles (Fig. 3B and SI Appendix, Figs. S10 and S11). Importantly, the LH on one side was infected (SI Appendix, Fig. S11 G and H), and a considerable number of Orx/Hcrt and MCH neurons was virus-labeled, especially the former neurons (SI Appendix, Fig. S12A–J). There was no evidence for neuron loss in this case (SI Appendix, Fig. S12K–M), suggesting a relatively early-stage viral attack. The LC showed a similar degree of changes and infected neurons were seen also in other areas of sleep–wake regulating network, including the DRN/MRN and TMN.

**Discussion**

In this study we show that the H1N1 influenza A virus causes alterations in the sleep–wake pattern in Rag1−/− mice and targets multiple, distinct neuronal populations belonging to the distributed
system of vigilance regulation. Because Rag1−/− mice lack B and T cells, the observed effects did not involve adaptive autoimmune mechanisms. The study could, thus, disclose neuronal populations targeted by the infection in the absence of an efficient immune control.

Sleep–Wake Alterations in the Infected Mice. During the fourth week postinfection, we observed changes in the sleep–wake pattern and features of sleep spectral power similar to those reported in murine models of narcolepsy, represented by Orx/Hcrt- and Orx/Hcrt receptor-deficient mice (31, 33, 36), indicating a fragmentation of vigilance states. Thus, during the dark/activity phase there was an inability to maintain consolidated wakefulness, which is a hallmark of human narcolepsy (30), described also in murine narcolepsy models (31, 33, 36, 37). Frequent sleep episodes interrupting wakefulness, as documented in our study after influenza virus infection, occur in Orx/Hcrt-deficient mice, which also exhibit an increased sleep time during the dark phase (31). In addition, a severe fragmentation of sleep, frequently interrupted by short episodes of wakefulness, was here observed in the infected mice during the light/rest phase. This is also an important element of the narcolepsy phenotype in humans (30), although this phase is less affected than the dark/activity phase in murine models of narcolepsy (31, 36, 37). In the present study,

Fig. 5. Viral antigens in the LH. (A–G) The LH is strongly infected and many Orx/Hcrt+ neurons are lost (see, for example, dashed areas on left and right side) (A), as is also evident in the adjacent Cresyl violet-stained section (E and F). However, viral antigens are still found in some Orx/Hcrt+ neurons in the surrounding, marginal area (B). The intermingled MCH+ neurons do not appear overtly affected (C), even if some are infected (D). (H) Triple labeling of virus with GAD67 and VGLUT2 shows a distinct network of nerve endings within the heavily infected area, although less dense in the infected side (Insets). (I) Double labeling with Iba1 shows activated microglia in the area (marginal area between circle and dashed circle, as indicated by arrowhead) surrounding the heavily infected area (for details, see SI Appendix, Fig. S8 A–C). (J) Astrocytes (GFAP+) are also activated but only in the marginal area (as labeled in I). Images in B and D were merged from z-stack (8-μm and 12-μm thick, respectively). (Scale bars: 500 μm in A, C, H, I, J; 20 μm in B and D; 500 μm in E; 100 μm in F and G.)
Fig. 6. Viral antigens in extrahypothalamic regions of the sleep-regulating network (arrowheads indicate colocalization). (A–D) Cholinergic neurons (choline acetyltransferase, ChAT) in the nucleus of the vertical limb of the diagonal band (VDB) (A and B) and histaminergic neurons (histidine decarboxylase, HDC) in the ventral tuberomammillary nucleus (VTM) (C and D). (E and F) Dopaminergic neurons (tyrosine hydroxylase, TH) in the VTA. (G and H) Serotoninergic (tryptophan decarboxylase, TPH) in the dorsal raphe (DR). Open arrowhead points to a nonserotoninergic neuron. (I–N) Noradrenergic neurons (TH) in the LC showing loss of TH compared with control. Adjacent Cresyl violet-stained section also shows neuronal loss (K–M) (see, for example, control in N). (O and P) Triple labeling (GAD67 plus VGLUT) shows that many nerve endings remain in the degenerated area ( Insets). (Q–S) Higher magnification images show virus-targeted neurons in LC (A and H) and outside LC: lateral dorsal tegmental nucleus (LDTg, Q), Barrington nucleus (Bar) and mesencephalic trigeminal nucleus (Me5, S). (T) Cartoon shows projections from the LC to OB allowing retrograde transport to NA neurons in the LC and presumably followed by local spread to surrounding neurons. Images were merged from z-stack: 17 μm (A and H), 6 μm (D), 14 μm (F), 13 μm (Q–S), respectively. (Scale bars: 500 μm in A, E, I, J, O, and P; 20 μm in B, D, F, and Q–S; 100 μm in C and G; 20 μm in H; 500 μm in K; 100 μm in L–N; 10 μm in Insets of O and P.)
the total amount of wakefulness, SWS, and REM sleep was not significantly altered in the infected mice during the light/rest phase, similarly to observations in Orx/Hcrt-deficient mice (31, 38). Therefore, importantly, in the infected mice there was no increase in the time spent in SWS, as it occurs in sickness behavior as a result of pulmonary influenza virus infections (20), and the increase in state transitions was not correlated to the sign of sickness represented by body weight loss.

The sleep–wake pattern thus showed narcolepsy-like alterations, including SOREM episodes, indicating imbalances in the network of sleep–wake regulatory neurons. In murine models of narcolepsy these include behavioral arrests, providing evidence of cataplexy by video recording (32), which could not be performed here because of the experimental conditions. Using our accelerometer recording method, we could document behavioral immobility during SOREM episodes, “cataplexy-like” episodes, thereby strongly supporting the EEG evidence of sleep–wake changes that define human narcolepsy per se (39) and, as discussed above, have been repeatedly reported in murine narcolepsy due to deficient Orx/Hcrt signaling.

**Influenza Virus Targets Olfactory Epithelium and Sleep–Wake Regulatory Neurons.** Previous observations have shown that neurons in the olfactory epithelium can be targeted by influenza A virus in mice (29), ferrets (24), and humans (40), and that H5N1 avian influenza strains in ferrets can spread along olfactory and trigeminal nerves to the OBs and brainstem after intranasal infection (24). In humans, influenza virus antigens in neurons and glia in the OBs and tracts, and the gyrus rectus have recently been found in an immune-compromised child (41).

By using Rag1–/– mice, which, as mentioned above, allow us to investigate primary effects of influenza A virus from the brain (29), we could follow the spread of the virus to nerve cell groups that project to the OB. Of particular interest, our study showed the presence of influenza A virus antigens in hypothalamic neurons, which produce Orx/Hcrt or MCH (42, 43) and are part of the sleep–wake regulatory network. These peptidergic neurons innervate the mitral and granule cell layers of the OB (44, 45), and thus represent a target for retrograde axonal transport of viruses along the olfactory pathway connectivity (46) (Fig. 7).

In addition, infected neurons were found in the basal forebrain, TMN, VTA, DRN/MRN, and LC, which contain cholinergic, histaminergic, dopaminergic, serotonergic, and noradrenergic neurons, respectively. These neuron groups, except the VTA, have been shown to project directly to the OB (47, 48) and are involved in the control of sleep and wakefulness (25–27).

In some of the infected mice the number of Orx/Hcrt-immunolabeled neurons was markedly reduced on the infected side, whereas the part that only intermingled with MCH– ones appeared better preserved. A possible differential susceptibility to the infection could reflect variations between neuronal populations with regard to viral uptake from their projection fields; alternatively, the known high vulnerability of Orx/Hcrt neurons (e.g., to nitric oxide-mediated S-nitrosation) could play a role (49). Postmortem examination of brains of human narcoleptics showed number of Orx/Hcrt-immunolabeled neurons to be dramatically reduced, whereas MCH-immunolabeled neurons were preserved (1, 2). However, it is difficult to determine to what extent our cell counts, showing differences between Orx/Hcrt and MCH neurons in the infected mice, are relevant for the above differences in the pathology of narcolepsy in humans.

In conditional Hcrt knockout mice, about 95% of Orx/Hcrt neurons are lost before cataplexy appears (33), and such a marked cell loss was not seen in our study. Taken together, our findings indicate that in the present paradigm the sleep pattern changes are not primarily because of cell death in a single cell group, such as the Orx/Hcrt neurons. Instead other mechanisms could be involved, such as cellular dysfunctions resulting from viral proteins such as the multifunctional nonstructural (NS1) protein (50) or the influenza nucleoprotein (NP) that accumulates in dendritic spines to reduce excitatory synaptic activity (51). Interestingly, antibodies to the NP can cross-react with human hypoxin receptor 2 (15), which may further interfere with Orx/Hcrt signaling. More modest injury/dysfunction in the multiple infected neuronal populations within the sleep–wake regulatory network may also play a role. In fact, little is known about the involvement of other than the Orx/Hcrt and MCH neurons in the sleep-wake regulating network in human narcolepsy (52). Furthermore, although systemic release of IL-1β and TNF-α is associated with hypersomnia, which was not here observed, a local release of such molecules into hypothalamic areas by activated glia may disturb sleep–wake regulations (53). The notion of noncytolytic effects on neurons by an infection is relevant also in view of the finding that not all narcolepsy patients have low Orx/Hcrt levels in the cerebrospinal fluid (14).

**Influenza Virus–Host Factor Interactions Involved in Neuroinvasion.** In general, the immune control of an infection in the nervous system is more efficient in peripheral ganglia (e.g., trigeminal ganglia) than in the CNS (46). Olfactory epithelium neurons are unique by bridging environmental exposure to pathogens with CNS tissue in the OBs, pointing to the OB as a critical portal for entry to the rest of the brain (Fig. 7). Because infections with influenza A viruses are usually limited to the respiratory epithelium,
it is important to determine factors that may control spread into adjacent nervous tissue.

Viral and host factors contribute to the pathogenesis of CNS involvement in influenza infections (54). Viral factors reside in both the envelope and the NS1 proteins to promote the neuropathology of certain influenza virus strains. For example, the surface glycoprotein neuraminidase is a major neurovirulence determinant of the presently used WSN/33 influenza virus strain (55). Changes in two amino acids in the NS1 of an H5N1 strain can shift the tropism of the virus from the lung to the brain (56). Interestingly, the NS1 protein can also regulate the host innate immune response and thus affect viral spread and tissue reactions (50). Variants of genetically related isolates of the H1N1pdm virus can induce specific host gene-expression profiles that differ with regard to innate immune response genes (57, 58).

In addition, acquired and genetic host factors affect the pathogenesis or outcome of influenza A virus infections (54). Studies of host genes in mouse models have identified a number of loci associated with susceptibility or resistance to severe influenza disease (59). However, little is known regarding the possible effects of human genetic variations, particularly in the HLA haplotypes, on the outcome of the infection with influenza virus strains (60).

The mouse-neuroadapted influenza A virus was used in this study. Influenza A/WSN/33 Virus Infection. The mouse-neuroadapted influenza A (A/WSN/33) virus strain was obtained from the National Institute of Allergy and Infectious Diseases (USA) (18) and used as the standard strain (19, 20). Influenza A/WSN/33 virus was grown in the allantoic cavity of 9- to 11-day-old embryonated chicken eggs (21) and titrated in 96-well plates using the method of Reed and Muench (22). The plaque test was performed to determine the presence of a single plaque-forming unit (23).

Materials and Methods

Animals. Female knockout mice (3- to 5-mo-old) for the Rag1−/− (background strain C57BL/6j) and wild-type mice were used. The experiments were approved by the local ethics committee (Stockholms Norra Djurförsöksstiftelse Nämnd, project N87/12).

Influenza A/WSN/33 Virus Infection. The mouse-neuroadapted influenza A virus was delivered intranasally in a volume of 20 μl per mouse (SI Appendix, Materials and Methods).

Surgery. Surgery was performed to implant wireless NeuroLogger microchips (Neurobehavior, TSE) to record EEG as well as body movement (actimetry) via a built-in accelerometer (SI Appendix, Materials and Methods).

EEG Recording and Analysis. NeuroLoggers were configured using specialized software (CommSw, Neurobehavior) and sampled at 199.8 Hz. Following baseline recordings, performed 7 d after surgery, the mice were treated intranasally with saline or infected and recordings were analyzed every week (SI Appendix, Materials and Methods).

Quantitative Real-Time RT-PCR. The quantitative PCR reactions were perfected with custom-designed primers on an ABI Prism 7000 real-time thermocycler (Applied Biosystems) (SI Appendix, Materials and Methods). Primers used in this study are listed in SI Appendix, Table S1.

Immunohistochemistry. For immunohistochemistry, 4% (wt/vol) paraformaldehyde-fixed brains were frozen and sectioned on a cryostat. Sections were processed using the TSA Plus method (PerkinElmer) and indirect Coons procedure (SI Appendix, Materials and Methods). Antibodies used in this study are listed in SI Appendix, Table S2.

Microscopy and Imaging. The material was examined with an LSM700 confocal laser-scanning microscope (Zeiss), and images acquired from one airy unit pinhole as previously described (SI Appendix, Materials and Methods).

Statistics. Statistical analyses were performed with SPSS (IBM). In all analyses, statistical significance was set at P < 0.05 (SI Appendix, Materials and Methods).
6. Mignot E, et al. (2001) Complex HLA-DR and -DQ interactions confer risk of narco-