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# Selective targeting of habenular, thalamic midline and monoaminergic brainstem neurons by neurotropic influenza A virus in mice

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**Infections caused by influenza A virus have been proposed to be associated with neuropsychiatric complications, the mechanisms of which remain to be unravelled. We here report that a neurotropic strain of influenza A virus (A/WSN/33) introduced into the olfactory bulbs of C57BL/6 (B6) mice, selectively attacks habenular, paraventricular thalamic, and brainstem monoaminergic neurons. In the habenular and paraventricular thalamic areas, infection was followed by an almost total loss of neurons within 12 days. In the brain stem monoaminergic areas, viral gene products were eliminated from neurons by 12 days in B6 wildtype mice, but remained for at least 35 days in immunodefective TAP1 (Transporter associated with Antigen Presentation 1) mutant mice. In conclusion, we show that influenza A virus infection in the brain selectively targets regions which have been implicated in neuropsychiatric disturbances, and that this virus can remain for a significant period of time in specific regions of the brain in immunodefective mice.**

**Keywords:** nervous system; behaviour; viral persistence; olfaction; limbic system; axonal transport

## Introduction

Infections with influenza A viruses in humans have often been associated with a number of neurological complications ranging from acute encephalitis and encephalopathy to a suspected relationship with long-term progressive neurodegenerative diseases and neuropsychiatric disturbances (for review see Murphy and Webster, 1996). However, little information is available on the pathogenesis of nervous system dysfunctions caused by influenza A virus infections.

Neurotropic influenza A virus strains can replicate in the mouse brain, and genes encoding for hemagglutinin, neuraminidase, matrix, as well as non-structural proteins, have all been shown to be important determining factors for the neurovirulence of this virus (Ward, 1996). The olfactory route of virus invasion of the brain is of interest, in particular, as the nasal cavity is the only site where neurons are directly exposed to the external environment. The access through olfactory bulb connections may convey the virus to both the limbic system and to the momoaminergic brainstem neurons. In experimental animals, a variety of viruses can be transferred by axons in both anterograde and retrograde directions in the olfactory system, with different areas targeted selectively by different viruses (for reviews see Mohammed *et al*, 1993; Kalicharran and Dales, 1996). Some of these infections are lethal, while others are cleared within a short period of time. Animals surviving non-lethal infections may be left with permanent behavioural changes and neurotransmitter disturbances in the brain (Mohammed *et al*, 1990; Andersson *et al*, 1993). A lethal infection attributed to the propagation of a highly neurovirulent recombinant between a human and an avian influenza A virus along olfactory and trigeminal pathways into the mouse brain has been described (Reinacher *et al*, 1983). However, the potential targets in the brain for influenza A virus after this route of entry have not been determined, and a model of a non-lethal infection has not been established.

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In the present study, we examined in B6 wildtype and immunodefective TAP1−/− mice (Van Kaer *et al*, 1992) the targeting and elimination of the neurovirulent A/WSN/33 influenza A virus strain (Takahashi *et al*, 1995), as well as the distribution of virus-induced lesions in the brain after stereotactic olfactory bulb injections. We here demonstrate that the virus can cause a non-lethal infection with a selective involvement of regions in the brain implicated in neuropsychiatric disturbances, and that the virus can remain in brainstem monoaminergic neurons for a significant period of time in immunodefective TAP1−/− mice.

## Results

Wildtype (B6) and TAP1−/− mice were injected stereotactically into the right olfactory bulb with the influenza virus A/WSN/33. Infected mice failed to gain weight between 3 and 12 days p.i. Otherwise, there were no overt clinical signs of infection and seizures were not provoked by the tail test. At 3 days p.i., a large number of influenza A virus infected cells were observed in the central part of the granular layer of the ipsilateral olfactory bulb in both B6 wildtype and TAP1−/− mice. From these infected neurons, numerous infected fibres radiated out into the mitral cell and external plexiform layers.

Within 1 week, the virus had spread into the cerebral ventricles from the injection site in the olfactory bulbs to infect ependymal cells. The medial habenular and paraventricular thalamic nuclei were then heavily infected with immunopositivity for viral antigens in almost all neurons (Figures 1 and 2a and b). Both these nuclei are located directly underneath the ependyma of the third ventricle and appeared to be vulnerable to the effects of the viral infection. In the brain parenchyma, neurons were the primary target of attack (Figure 3).

The virus was also targeted to neurons, which project from the brain to the olfactory bulbs (Shipley and Adamek, 1984). Thus, clusters of infected neurons were seen bilaterally in the ipsilateral anterior olfactory nuclei (AON), the ipsilateral piriform cortex, and the midbrain raphe nuclei (Figures 1 and 2c) as well as in the locus coeruleus at 7 days p.i. Some infected neurons were also observed in the ipsilateral hypothalamus and at the periphery of the posterior mammillary nuclei. Furthermore, several dopaminergic neurons were infected in the ipsilateral ventral tegmental area, but not the substantia nigra, as revealed by double immunolabelling of virus antigens and tyrosine hydroxylase, which is a marker for dopaminergic neurons. Immunopositivity for viral antigens was confined to the neurons (Figure 4).

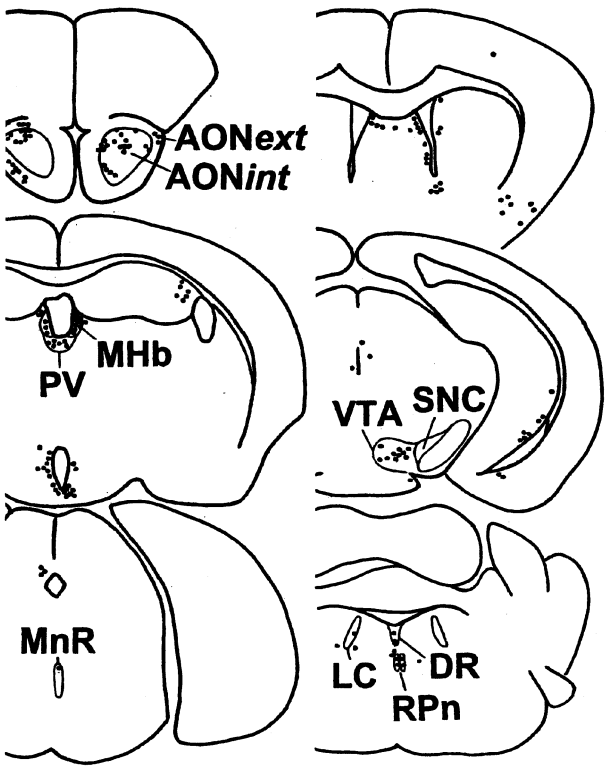


**Figure 1** Distribution of influenza A virus immunopositive neurons in the brain of a TAP1−/− mouse 7 days p.i. (AON, Anterior olfcatory nuclei; MHb, medial habenula; PV, paraventricular nucleus; VTA, ventral tegmental area; SNC, Substantia nigra, compact; MnR, median raphe; DR, dorsal raphe; Rpn, raphe pontis nucleus; LC, locus coeruleus).

In the above-described general pattern of infection, there were no differences with respect to viral spread and localisation between wildtype and TAP1−/− mice within the first week of infection. However, at 12, 18 and 35 days p.i., there was a marked difference in the occurrence of viral antigens in the brain. TAP1−/− mice displayed numerous infected monoaminergic brainstem neurons, particularly in the ipsilateral ventral tegmental area (Figure 2d), in the dorsal and medial raphe nuclei, and occasionally in the locus coeruleus. Infected neurons were also abundant in the AON; isolated or small clusters of infected neurons were seen in the rostral part of the ipsilateral piriform cortex. In contrast, the wildtype mice showed no infected neurons at these time points (Figure 5). In both B6 wildtype and TAP1−/− mice, there was an almost total neuronal loss in the habenular and the paraventricular thalamic nuclei 12–35 days p.i. (Figure 6). In the other areas of infection, nerve cell loss was less evident.

Although infectious virus was recovered from the brains 7 days p.i., no infectious virus could be detected in either type of mice at subsequent time

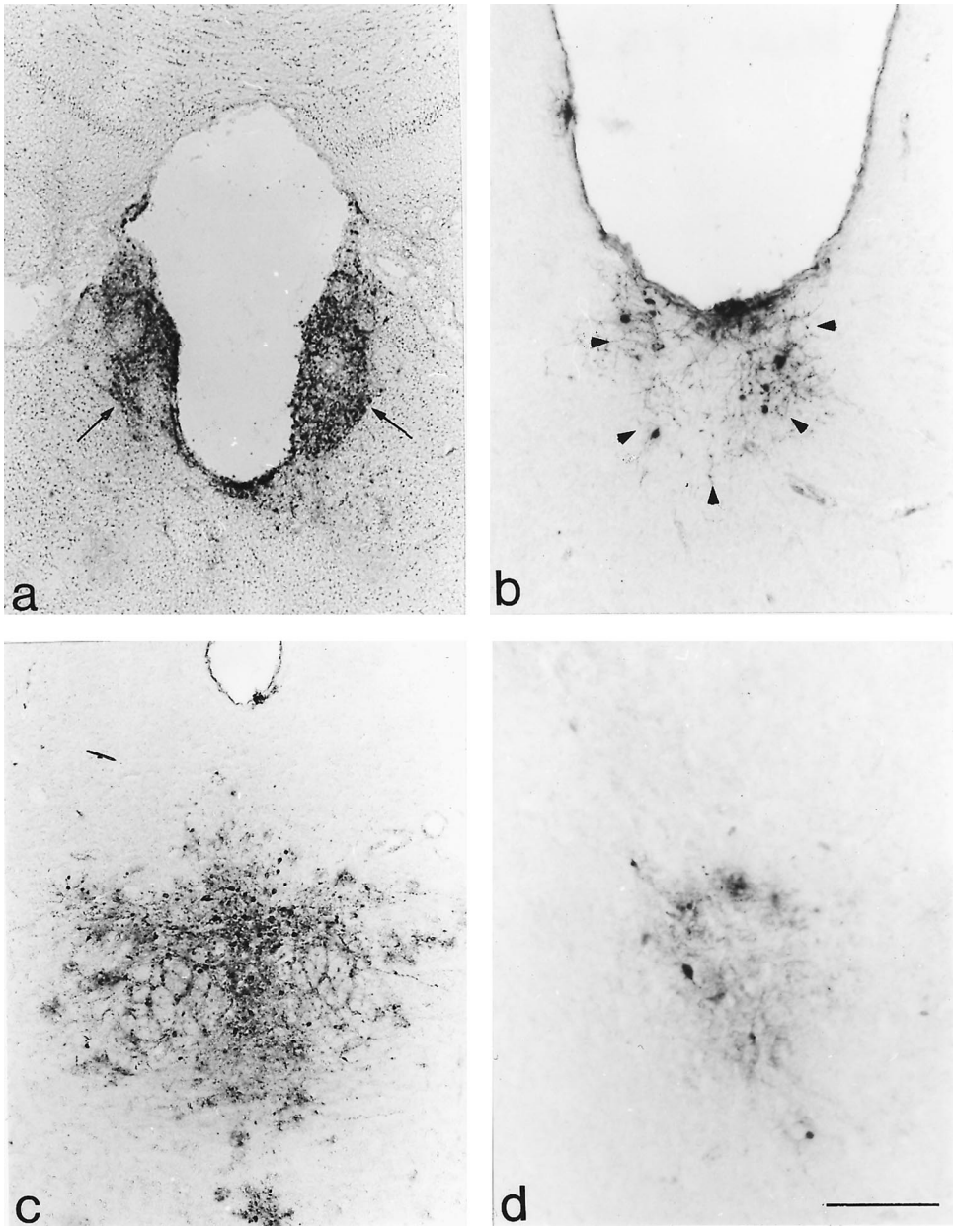


**Figure 2** Immunohistochemical analysis of influenza A virus antigens in the brain. (**a**) The medial habenular nuclei (arrows) of a TAP1−/− mouse 7 days p.i.; (**b**) the paraventricular thalamic nucleus (arrowheads) of a wildtype mouse 3 days p.i.; (**c**) the dorsal raphe nucleus of a TAP1−/− mouse 7 days p.i., and (**d**) the ventral tegmental area of a TAP1−/− mouse 18 days p.i. Scale bars: (**a**) and (**c**), 400 $\mu$m; (**b**) and (**d**), 200 $\mu$m.

points (Figure 7); the minimal virus titer detectable has been estimated to 20 p.f.u. per brain (Takahashi *et al*, 1995). Presence of viral RNA in brains of the mice was verified by semi-nested reverse transcription-PCR. Viral sequences of NS1 and NP were detected 12 days p.i. in each of two brains examined from both wildtype and TAP1−/− mice. At 18 days p.i., both brains and at 35 days p.i. one of two brains from TAP1−/− mice were positive (Figure 8). In wildtype mice viral sequences were detected in two of four brains at 18 days p.i., but in none of four brains at 35 days p.i. The restriction digestion pattern of the amplified NP verified the expected restriction site in the sequence.

Infiltration of lymphocytes was evaluated in sections through the ipsilateral AON. At 3 days p.i., $CD4^+$ T cells were seen in the infected areas in both wildtype and TAP1−/− mice. Presence of $CD4^+$ T cells was documented throughout the sampled times (7, 12, 18 and 35 days p.i., respectively) in both strains of mice. As expected, marked differences were observed with respect to

presence of CD8+ T cells in wildtype and TAP1−/− mice. CD8+ T cells were not detected at any time point in TAP1−/− mice. In contrast, few CD8+ T cells were observed in infected areas in wildtype mice at 3 days p.i.; at 7, 12, 18 and 35 days p.i., infiltration of CD8+ T cells was pronounced (Figure 5). Thus, both wildtype and TAP1−/− mice were able to mount a CD4+ T cell response, whereas a CD8+ T cell response was only detected in wildtype mice. This observation correlates with the findings of persistence of influenza virus antigens 35 days p.i. in neurons of the TAP1−/− mice. The present observations therefore support the notion that CD8+ T cells are important for clearance of the neurotropic influenza A virus from neurons in the brain as demonstrated by Stevenson *et al* (1996) and Hawke *et al* (1998).

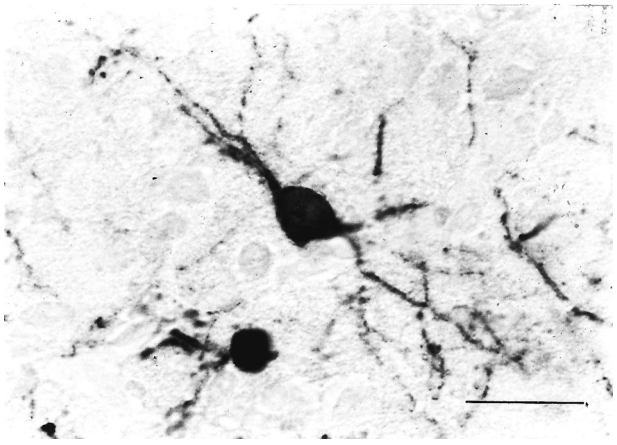

**Figure 3** Immunolabelling of viral antigens in cell bodies and dendrites of periventricular neurons 3 days p.i. Scale bar: 40 $\mu$m.

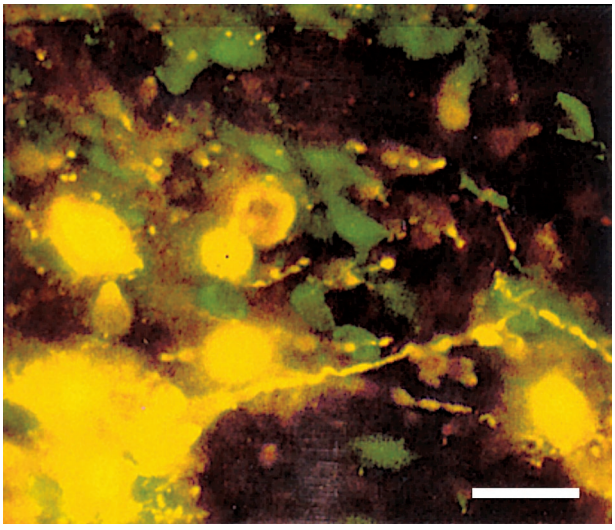

**Figure 4** Double immunolabelling of influenza A virus antigens and dopaminergic neurons. Anti-tyrosine hydroxylase antibodies label the neurons green. Co-localisation of tyrosine hydroxylase and viral antigen (yellow) occurs in several neurons of the ipsilateral ventral tegmental area. TAP1−/− mouse 7 days p.i. Scale bar: 40 $\mu$m.

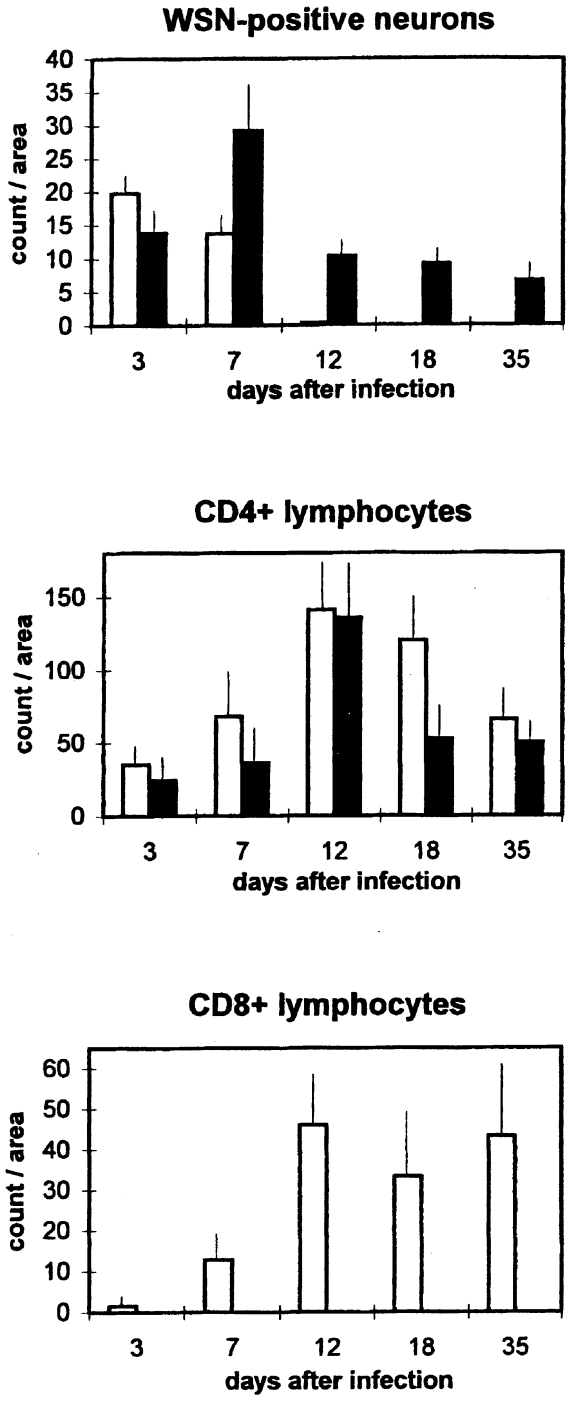


**Figure 5** Viral antigens and T lymphocytes in the AON. (**a**) Influenza A virus-immunopositive neurons, (**b**) CD4+ T cells and, (**c**) CD8+ T cells. Immunoreactive cells were counted in the whole field of the right AON. Wildtype (empty bars) and TAP1−/− (filled bars) mice. Data are shown as mean ± s.e.m. ($n$=6 mice at each time point).

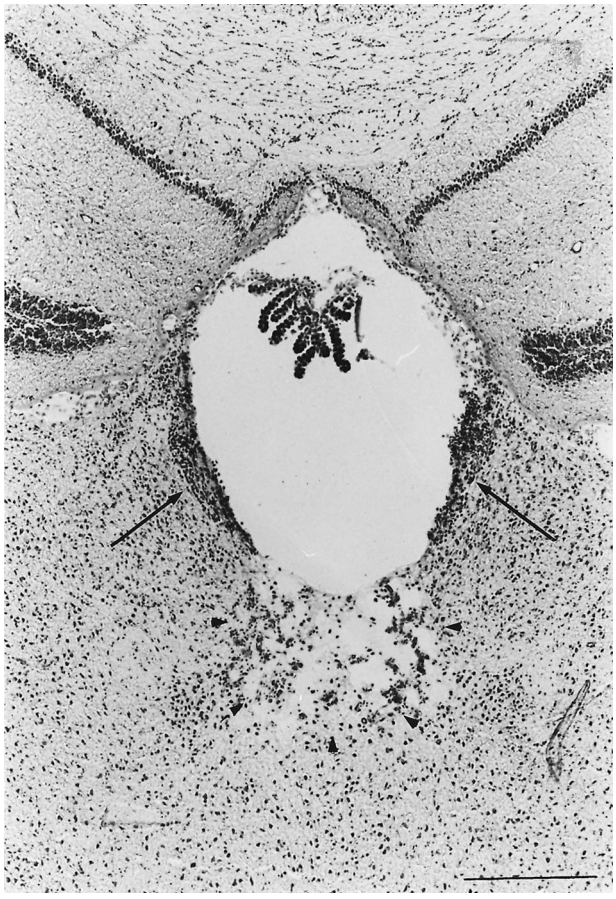

**Figure 6** Selective lesions caused by the virus. Section through the brain of a TAP1−/− mouse, 35 days p.i., showing lesions of the medial habenula (arrows indicate remnants of the nuclei) and paraventricular thalamic nucleus (arrowheads outline the destroyed nucleus). Cresyl-violet acetic-acid. Scale bar: 400 $\mu$m.

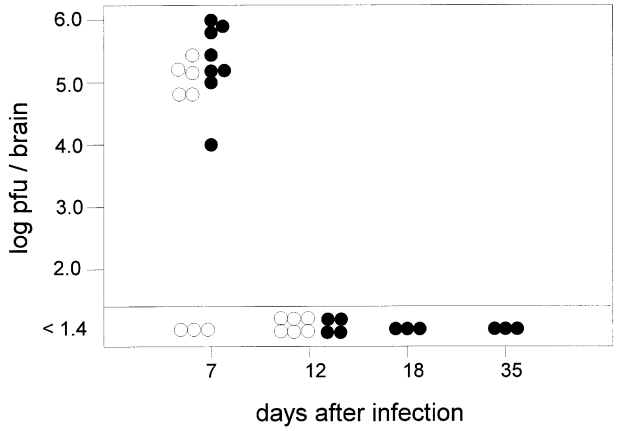


**Figure 7** Titers of infectious influenza A virus in the brain of wildtype (open circles) and TAP1−/− mice (filled circles) after olfactory bulb injection.

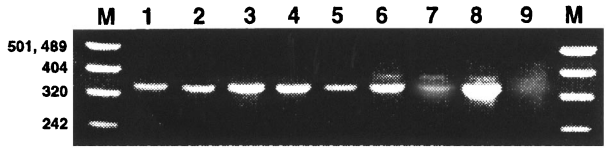


**Figure 8** Semi-nested RT−PCR analysis of NP RNA from influenza A infected wildtype and TAP1−/− mouse brains. Lanes M, molecular weight markers (mixture of pUCBM21 DNA digested with *Hpa*II and pUCBM21 DNA digested with *Dra*I and *Hind*III); lanes 1 and 2, brains from wildtype mice 12 days p.i.; lanes 3 and 4, brains from TAP1−/− mice 12 days p.i.; lanes 5 and 6, brains from TAP1−/− mice 18 days p.i.; lane 7, brain from TAP1−/− mouse 35 days p.i.; lane 8, positive control (influenza A/WSN/33 grown in MDCK cells); lane 9, negative control (uninfected mouse brain). Amplified products were analysed on a 1% agarose gel containing ethidium bromide and visualised and photographed on exposure to UV light.

## Discussion

The present observations unequivocally point out that in mice influenza A virus has a high propensity to cause selective infection of neurons in the habenula, midline thalamus, ventral tegmental area and raphe, and that the infection can be long-lasting when host immune mechanisms are impaired.

A remarkable feature of the present study was an early selective and extensive infection of the medial habenular and the paraventricular thalamic nuclei. Such selective virus-induced lesions in the brain may have important functional implications. The habenular nuclei provide a link between limbic and striatal forebrain areas, and lower diencephalic and mesencephalic centres (Ellison, 1994). They may modulate functions such as sensory gating through the thalamus, and reward mechanisms through the dopaminergic neurons of the substantia nigra and ventral tegmental area. Experimental lesions of the habenula can produce a wide variety of behavioural alterations. Continuous exposure of rats to amphetamine or cocaine results in selective degeneration of the habenula, which may contribute to the disturbed 'late-stage' behaviour in such rats (Ellison, 1994). In the paraventricular thalamic nucleus, which is the most dorsal component of the thalamic midline nuclei, cell populations project to the nucleus accumbens, amygdala, and hippocampus (Su and Bentivoglio, 1990; Bentivoglio *et al*, 1991). Knowledge about the functional implications of these circuits is still scarce. However, the dendrites of neurons of the paraventricular thalamic nuclei, which project to the amygdala, have been shown to reach the ependymal lining (Balceria *et al*, 1992), and, thus, may provide a direct access of the viral infection from the ventricle to thalamo-limbic neurons. It is also of interest to note that the thalamic midline paraventricular nuclei can be selectively activated by antipsychotic drugs (Deutch *et al*, 1995; Cohen *et al*, 1998), indicating that they may represent a target of action of these substances.

Dopaminergic neurons of both the substantia nigra and the ventral tegmental area have been reported to be infected by the virus after direct inoculation in a cerebral hemisphere (Takahashi *et al*, 1995), that could have involved target areas of substantia nigra efferents, but also after hematogeneous spread of the virus (Yamada *et al*, 1996). The present finding of a confinement of the infection to neurons in the ventral tegmental area after virus injection into the olfactory bulbs confirms the susceptibility of dopaminergic neurons to influenza A virus infection and indicates a retrograde axonal transport of the virus, since these neurons project to the infected AON (Oades and Halliday, 1987). In contrast to previous observations on measles virus spread in TAP1−/− mice (Urbanska *et al*, 1997), neurons in the main target areas of olfactory bulb efferents (Shipley and Adamek, 1984) were not infected. This may reflect a sparse infection of neurons in the mitral cell layer, which project anterogradely to the brain (Shipley and Adamek, 1984). In the ventral tegmental areas, which were less destroyed than the habenula and paraventricular thalamic nuclei, infected neurons occurred still 35 days p.i. in the TAP1−/− mice. Although persistent infections with influenza A virus have been obtained *in vitro* (Tobita *et al*, 1997), this is to our knowledge the first description of a more long-term occurrence of influenza virus antigens in the brain.

Since the brain structures targeted by the influenza virus infection are implicated in the pathogenesis of neuropsychiatric disturbances, a potential role for the virus to cause such dysfunctions in humans may be speculated upon. Olfactory dysfunctions are common after influenza infections in humans (Henkin *et al*, 1975) and influenza virus can penetrate into the human brain to infect ependymal and periventricular cells (Frankova *et al*, 1977). A reversible schizophrenic syndrome was by far the most frequent psychiatric complication following the 1918–1919 influenza pandemic (Menninger, 1926). This influenza pandemic was followed by the global pandemic of *encephalitis lethargica*, estimated to have led to more than 1 000 000 cases of severe neurological disease during the ensuing decade (von Economo, 1929; Yahr, 1978). Although, circumstantial evidence has suggested influenza A virus as a potential cause of this pandemic (Maurizi, 1989; Ravenholt and Foege, 1982), such an opinion has been opposed and the true culprit(s) responsible for this specific syndrome remains unclear (Johnson, 1982). In this context, it should also be noted that a possible relation between prenatal exposure to influenza and different psychiatric disorders later in life is presently under debate (Machón *et al*, 1997). Our model of a non-lethal influenza A virus infection targeted to brain circuits implied in behaviour disturbances will be suitable to analyse in detail behavioural consequences of an attack by this respiratory virus.

## Materials and methods

Influenza virus A/WSN/33 was obtained from Dr S Nakajima (The Institute of Public Health, Tokyo, Japan) (Takahashi *et al*, 1995), and propagated on Madin-Darby canine kidney (MDCK) cell monolayers in MEM (Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine, 0.01 M HEPES buffer and 0.2% BSA (all obtained from Sigma, St. Louis, MI, USA) and penicillin G (50 IU/ml)/streptomycin (50 $\mu$g/ml) (Life Technologies). The virus suspension was titrated by a plaque assay in the absence of trypsin (Tobita *et al*, 1975). The virus titer was $5 \times 10^7$ p.f.u./ml.

Mice with targeted disruption of the TAP1 gene were derived from a breeding pair obtained from Dr L Van Kaer (Vanderbilt University, Nashville, TN, USA) (40). TAP1−/− were back-crossed to C57BL/6 (B6) mice at least six times before use. B6 wildtype and TAP1−/− mice were bred at the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm. Four-week-old mice, comprising an equal number of both sexes, were used for infection. Following anaesthetisation by the intraperitoneal administration of 7.2% chloral hydrate mice were placed in a stereotactic frame. Using a dentist's drill, a hole was made on the skull above the middle portion of the right main olfactory bulb. One $\mu$l of the virus suspension was injected by a Hamilton syringe, at a depth of 2.2 mm and at a rate of 0.2 $\mu$l/min. The needle was kept *in situ* for 5 min after injection to minimise leakage of the inoculum along the needle track. Control mice (four wildtype and four TAP1−/− each) were injected with 1 $\mu$l of MEM containing 2 mM L-glutamine, 0.01 M HEPES, and 0.2% BSA. Mice were weighed and evaluated clinically before infection and 3, 7, 12, 18, 25 and 35 days post infection (p.i.). This evaluation included ruffled fur, shivering, hunching and altered motor activity, and convulsions by tail rotation. All experiments were in line with institutional guidelines and have been approved by the local animal ethical committee.

Under deep anaesthesia the mice were perfused with ice-cold 4% paraformaldehyde in Sörensen's phosphate buffer. The brains were dissected, soaked in 15% sucrose in PBS at 4°C for 15–20 h and frozen. Coronal sections, 14 $\mu$m thick, were cut on a cryostat at levels encompassing the main olfactory bulbs, anterior olfactory nucleus, caudate/putamen, diencephalon, midbrain and brainstem at the pontine level.

Six B6 wildtype and six TAP1−/− mice were sampled at 3, 7, 12, 18 and 35 days p.i. each. For immunohistochemical detection of viral antigens, sections from each animal were preincubated for

10 min in 5% normal swine serum (DAKO, Glostrup, Denmark) in PBS containing 0.3% Triton X-100 (Sigma), and incubated for 1 h with rabbit polyclonal anti-WSN serum (a generous gift from Dr S Nakajima), diluted 1 : 1000 in 2% normal swine serum in PBS supplemented with 0.3% Triton X-100. The endogenous peroxidase activity was quenched by incubating the tissues in 0.3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 5 min. The sections were subsequently reacted in biotinylated swine anti-rabbit Ig (DAKO), diltuted 1 : 400 in 2% normal rat serum in PBS containing 0.3% Triton X-100 for 1 h, in avidin-biotinylated horseradish peroxidase (DAKO) for 30 min and finally in sodium-acetate buffer, pH 5.3, containing 0.02% 3-amino-9-ethylcarbazole (Sigma) and 0.02% hydrogen peroxide for 15 min. Following thorough washing, the sections were mounted in glycerol-gelatin. An adjacent series of sections was stained with cresyl violet-acetic acid for histological examination and cytoarchitectonic control.

For staining of lymphocytes the following antibodies were used: Rat monoclonal anti-CD8a (Ly-2) and anti-CD4 (L3T4) (53-6.7 and RM4-5, respectively; PharMigen, San Diego, CA, USA), both diluted 1 : 100. The sections were incubated with the primary antibodies for 48 h at 4°C. Secondary antibodies included biotinylated rabbit anti-rat Ig (DAKO), diluted 1 : 400. Binding was visualised as described above.

For double immunolabelling of virus antigens and tyrosine hydroxylase, brain slices were incubated in 5% normal swine serum containing 0.3% Triton X-100 for 10 min, followed by the rabbit anti-WSN antiserum diluted 1 : 200 in 2% normal swine serum containing 0.3% Triton X-100 for 24 h at 4°C, and mouse monoclonal antibodies to tyrosine hydroxylase (Incstar, Stillwater, MN, USA), diluted 1 : 1600, for 48 h at 4°C. The sections were then incubated with TRITC-conjugated swine anti-rabbit Ig (DAKO) for 30 min, and FITC-conjugated rabbit anti-mouse Ig (DAKO) for 30 min at 37°C, and finally mounted in PBS-buffered glycerin with 0.1% *p*-phenylene diamine. The double immunofluorescent labelling was visualised in a Nikon fluorescence microscope at excitation wavelengths 495 (FITC) and 546 (TRITC). A double absorption filter that allowed the simultaneous identification of the two different colours, was used.

Infected brains were examined for virus titers at 7, 12, 18 and 35 days p.i. The brains, including the olfactory bulbs, were dissected and stored at −80°C before examination. They were homogenised in 2 ml ice-cold MEM, supplemented with L-glutamine (2 mM), penicillin G (75 IU/ml)/streptomycin (75 $\mu$g/ml), by the use of a glass homogeniser. Tenfold dilutions were made in MEM. The plaques were allowed to form on MDCK cell monolayers as described above.

Total RNA from 100 $\mu$l of a 20% (in MEM) homogenate of influenza virus infected mouse brains was extracted by the acid phenol method (Chomczynski and Sacchi, 1987) and reverse transcribed using random hexamer primers and 100–200 units of superscript reverse transcriptase (Life Technologies, USA) at 45°C for 1 h and 70°C for 10 min. The total cDNA generated from each sample was then used for PCR amplification of the NS1 viral sequence using the following primers: nucleotides 141–168, 5′-GATCAGAAGTCCCTAAGAGGAAGAG-3′ (WS-8-1 sense) and nucleotides 460–484, 5′-TCTTCGGTGAAGGCCCTTAGTAAT-3′ (WS-8-2 antisense). The amplification of the NP viral sequence was performed using semi-nested PCR using the following primers for the initial PCR reaction: nucleotides 54-83, 5′-TCAAGGCACCAAACGATCTTACGAACAGAT-3′ (NP-54 sense) and nucleotides 627–656, 5′-CGATCATTGATCCCACGTTTGATCATTCTG-4′ (NP-656 antisense). For semi-nested PCR, a primer corresponding to nucleotides 309–338, 5′-TCCTAAGAAAACTGGAGGACCTATATACAGG-3′ (NP-309 sense) was used with primer NP-656 described above. PCR was carried out in a Perkin Elmer Gene Amp PCR System 24,000, in a buffer containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dNTP mix, 50 pM each primer, 2.5 units of ampliTaq Gold (Perkin Elmer) with the following thermal cycle program: 93°C for 9 min followed by 25–40 cycles of 94°C/40 s, 58–60°C/30 s, 72°C/45 s, and a final extension at 72°C for 7 min. In the semi-nested PCR, 25 cycles were performed in the first amplification step while in the second amplifcation step (semi-nested) one twentieth product from first amplification step was amplified for a total of 40 cycles using the semi-nested primers. PCR products were analysed on 1% agarose gels. The amplified product of the semi-nested PCR was purified from the agarose gel by Sephaglass extraction (Amersham Pharmacia Biotech, Sweden) and digested with the *Bam*HI restriction enzyme to confirm the specificity of the product. The *Bam*HI restriction site is present at position 522 in the restriction map of NP, which is between the primers NP-309 and NP-656.

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