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In vitro demonstration of neural transmission of avian influenza A virus

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Neural involvement following infections of influenza viruses can be serious. The neural transport of influenza viruses from the periphery to the central nervous system has been indicated by using mouse models. However, no direct evidence for neuronal infection has been obtained *in vitro* and the mechanisms of neural transmission of influenza viruses have not been reported. In this study, the transneural transmission of a neurotropic influenza A virus was examined using compartmentalized cultures of neurons from mouse dorsal root ganglia, and the results were compared with those obtained using the pseudorabies virus, a virus with well-established neurotransmission. Both viruses reached the cell bodies of the neurons via the axons. This is the first report on axonal transport of influenza A virus *in vitro*. In addition, the role of the cytoskeleton (microtubules, microfilaments and intermediate filaments) in the neural transmission of influenza virus was investigated by conducting cytoskeletal perturbation experiments. The results indicated that the transport of avian influenza A virus in the neurons was independent of microtubule integrity but was dependent on the integrity of intermediate filaments, whereas pseudorabies virus needed both for neural spread.

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INTRODUCTION

Influenza A viruses belong to the family *Orthomyxoviridae* and contain eight single-stranded, negative-sense RNA segments that encode 10 polypeptides. Influenza A viruses are divided into subtypes on the basis of serological and genetic differences in their surface glycoproteins, haemagglutinin and neuraminidase, and the genes that encode them. Fifteen subtypes of haemagglutinin and nine subtypes of neuraminidase have been identified and all can be obtained from their natural hosts, water fowl and shore birds (Kida *et al.*, 1980, 1987; Webster *et al.*, 1992). Most influenza infections in humans cause upper respiratory tract disorders, but the other organs including the lungs, heart, liver, kidneys, muscles and brain can also be affected (Turner *et al.*, 2003). Central nervous system (CNS) involvements can be fatal particularly in infants and children (Togashi *et al.*, 1997), or pregnant women (Hakoda & Nakatani, 2000). Influenza A viruses or their genomes have been detected in cerebrospinal fluid (Hakoda & Nakatani, 2000; Togashi *et al.*, 1997) and viral proteins have been found in brain tissues (Frankova *et al.*, 1977).

In the 20th century, many influenza epidemics and pandemics have been caused by H1N1, H2N2 and H3N2 viruses. From 1997, H5N1 infections occurred in Asian countries and resulted in high mortality (Centers for Disease Control & Prevention, 2004; Tran *et al.*, 2004; Yuen *et al.*, 1998). Although it is thought that these viruses

were derived directly from avian species, and person-to-person spreads were rare and inefficient (Claas *et al.*, 1998; Mounts *et al.*, 1999). The emergence of H5 viruses in humans has proved that avian influenza viruses can cross the host species barrier and warns of the threat of the pandemic of new human influenza A viruses resulting from the mutation or reassortment of human influenza viruses with co-infecting avian influenza A viruses.

Previously, we have established mouse models in which intranasally inoculated H5 influenza A viruses afferently infected the CNS via transneural routes (Matsuda *et al.*, 2004; Park *et al.*, 2002; Shinya *et al.*, 1998, 2000; Tanaka *et al.*, 2003). Here, we demonstrate retrograde axonal transport of a neurotropic H5 influenza virus using neurons in a compartmentalized culture system, and evaluate the effects on viral spread in the presence of drugs interfering with microtubules (MTs), microfilaments (MFs) and intermediate filaments (IFs), making comparisons with pseudorabies virus, a neurotropic alphaherpesvirus which can propagate transaxonally *in vitro* and *in vivo* (Card *et al.*, 1990; Enquist *et al.*, 2002; Field & Hill, 1974).

METHODS

Viruses. Avian influenza virus strain 24a5b (AIV), a highly pathogenic strain, was obtained from the avirulent strain A/whistling swan/Shimane/499/83 (H5N3) after 24 consecutive passages in the

air sacs of chicks and five passages in the brain of chicks; and has a serial basic amino acid sequence at the haemagglutinin cleavage site (Ito *et al.*, 2001). AIV exhibits marked pathogenicity in chicks (100% mortality for 3-day-old chicks on air sac infection) (Silvano *et al.*, 1997) and neuropathogenicity in mice on intranasal inoculation (Matsuda *et al.*, 2004; Shinya *et al.*, 1998, 2000). This virus does not cause any pathological changes due to haematogenous viral spread in mice (Shinya *et al.*, 2000). AIV was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. All virus infection and incubation experiments were undertaken in the P3 biosafety area of our facility.

Pseudorabies virus, also known as Aujeszky's disease virus or suid herpes virus 1, belongs to the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*. Pseudorabies virus strain Yamagata-S 81 (PRV) is the first Japanese isolate from piglets (Itakura *et al.*, 1981). This virus was propagated in cloned porcine kidney (CPK) cells.

Neuron culture. Sensory neurons from the dorsal root ganglia (DRG) of the spinal cord of newborn BALB/c mice (2–4 days old) were dissociated by incubation with 1 mg collagenase (Sigma-Aldrich) ml⁻¹ at 37 °C for 30 min. Dissociated neurons were resuspended at a concentration of 40 000 neurons (about 80 ganglia) per ml in maintenance medium (MM) comprising Eagle's minimal essential medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 50 U penicillin (Invitrogen) ml⁻¹ and 50 µg streptomycin (Invitrogen) ml⁻¹. Collagen-coated 35 mm dishes with a central 14 mm glass coverslip (Matsunami Glass) were seeded with 100 µl of the cell suspension. Cells were then incubated in MM with 10 µM 5-fluoro-2'-deoxyuridine (Sigma-Aldrich) and 40 nM 2.5S nerve growth factor (Invitrogen) in a 5% CO₂ atmosphere at 37 °C. For the elimination of dividing cells, 6 µM aphidicolin (Sigma-Aldrich) was added to the medium from days 2 to 3 in addition to fluorodeoxyuridine. The medium was changed every 2–3 days. After 7–10 days of incubation, when an axonal network of neurons had formed, dishes were used for the experiments. At this time, over 1600 neurons survived in each dish and less than 3% of all surviving cells were non-neuronal cells, i.e. Schwann cells and fibroblasts which were identified by morphology and immunocytochemistry (data not shown).

Infectious titre in neurons. To detect the infectious titre of stock viral suspensions in neurons, both AIV and PRV were diluted 10-fold in MM and overlaid on cultured neurons. After 60 h incubation in a 5% CO₂ atmosphere at 37 °C, cells were screened for infectivity by immunofluorescent staining. Infectivity was calculated as 50% neuronal tissue culture infective dose (NTCID₅₀). Neuronal infectious titres of stock suspensions of AIV were 10^{5.7} NTCID₅₀ ml⁻¹, which was 10^{7.5} 50% egg infectious doses per ml, and that of PRV were 10^{7.1} NTCID₅₀ ml⁻¹, 10^{7.7} TCID₅₀ ml⁻¹ in CPK cells, respectively.

Infection of neuronal cell bodies with AIV. Cultured neurons in the 14 mm coverslip were inoculated with AIV at an NTCID₅₀ of 10^{2.7} in 100 µl MM and incubated for 1 h at 37 °C. The viral suspension was removed and cells were washed three times with MM, then incubated in MM for 6, 12, 18, 24 and 36 h. At least three samples were examined for each time point. Mock-infected neurons were used as a negative control.

Compartmentalized neuron culture. The culture dishes for the compartmentalized culture system were prepared as follows (Fig. 1, Campenot, 1977; Campenot & Martin, 2001): 35 mm dishes with a central 27 mm glass coverslip (Matsunami Glass) were coated with collagen. Parallel scratches were made by fine needles on the coverslip. A drop of MM containing 0.4% methyl cellulose was placed on the scratched region. A silicon divider (Shin-Etsu Chemical) with one end coated with a ribbon of silicon high vacuum grease (Dow

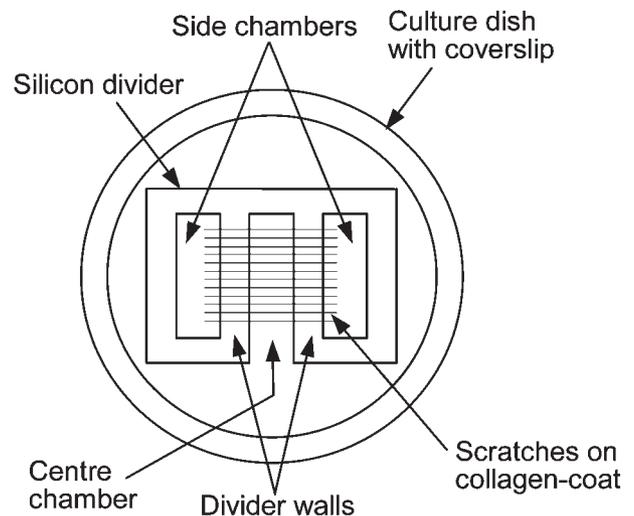


Fig. 1. Schematic diagram of a compartmentalized culture consisting of a silicon divider set on a 35 mm culture dish with a central 27 mm coverslip. A silicon divider with silicon high vacuum grease at the base is seated on a collagen-coated coverslip. Neurons seeded in the central chamber extend their axons along the scratches and reach the side chambers.

Corning) was placed on the coverslip so that each chamber was sealed off. The wet surface of the scratched region with the medium prevented the silicon grease from adhering to the collagen, and neurons seeded in the central chamber could extend their axons to the side chambers through the gap between the silicon divider and collagen bed without any exchange of medium between the chambers. No leakage of the medium from the chambers was checked at each change of medium. Samples of resuspended cells (2000 neurons in 50 µl MM) were seeded into the central chamber. Growing axons penetrated under the divider walls and when the total number of axons in both chambers reached 20, each dish was used for viral inoculation. At this time, over 800 neurons survived in each central chamber and less than 3% of all surviving cells were non-neuronal cells.

Inoculation of axons in compartmentalized culture system.

We inoculated AIV or PRV into the axons of the side chambers. Before the inoculation, the culture medium of the central and side chambers was removed, and MM containing rabbit antiserum against each virus and 0.2% methyl cellulose was added to the central chamber. Antiserum against influenza virus A/duck/Pennsylvania/10128/84 (H5N2) or against PRV (a gift from Dr Ono, Institute for Genetic Medicine, Hokkaido University, Japan) was used at a dilution of 1:20 for AIV and 1:50 for PRV. The antisera were used to neutralize viruses that leaked from the side chambers during absorption and extracellular viruses released from neurons. The concentrations of antiserum were enough to neutralize the viruses inoculated directly into the MM of the central chamber. For the viral infection of axons, AIV at an NTCID₅₀ of 10^{3.6} or PRV at an NTCID₅₀ of 10^{4.0} in 80 µl MM was inoculated in the side chambers and incubated for 1 h at 37 °C. The side chambers were then washed three times with MM and the medium was replaced with MM containing each antiserum and 0.2% methyl cellulose. At 6, 12, 18, 24, 36, 48 and 60 h post-inoculation (p.i.), dishes were used for immunofluorescent staining. At least three dishes were examined for each virus and for each time point.

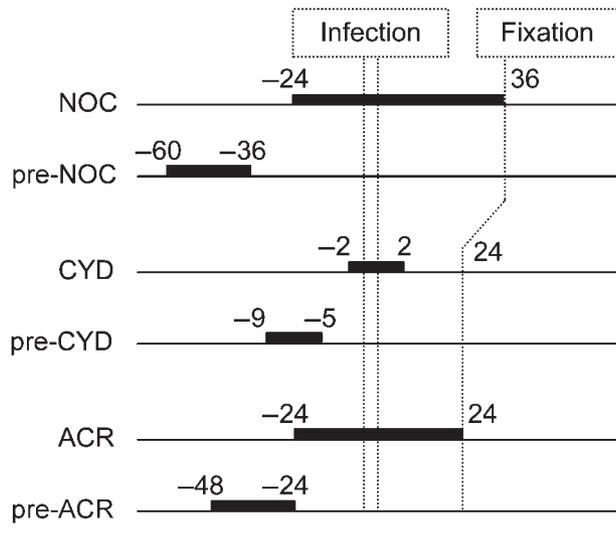


Fig. 2. Time schedule of drug treatment and viral infection. Neurons were infected with influenza virus strain 24a5b or pseudorabies virus strain Yamagata S-81 during or after drug treatment (filled bars on each cross line). Numbers represent hours before or after viral infection. NOC, Nocodazole; CYD, cytochalasin D; ACR, acrylamide.

Cytoskeletal interference and viral infection. Full-grown neurons with axonal networks were prepared on 14 mm coverslips on 35 mm dishes. Neurons pre-treated and recovered, or following treatment with 10 μ M nocodazole (NOC; Sigma-Aldrich), 5 μ M cytochalasin D (CYD; Sigma-Aldrich) or 4 mM acrylamide (ACR; Sigma-Aldrich), were inoculated with either AIV at an NTCID₅₀ of 10^{3.7} or PRV at an NTCID₅₀ of 10^{4.1} in 100 μ l MM and incubated for 1 h at 37 °C (Fig. 2). The inoculum was then removed by washing with MM three times and MM was applied with or without each drug. NOC was used for the disruption of MTs, CYD for the disruption of MFs and ACR for the perturbation of IFs. Stock solutions of NOC (10 mM) in DMSO, CYD (1 mM) in DMSO and ACR (1 M) in double-distilled water were prepared and diluted to a final concentration in MM. The final concentrations were chosen to maximize the effect without any apparent morphological cytotoxicity

(data not shown). Controls were treated with an equal volume of DMSO or double-distilled water. On the withdrawal of each drug, cells were washed with MM three times. At 24 or 36 h p.i., infected neurons were fixed for morphological examination. At least three dishes were used for each treatment. Student's *t*-test was used to assess the statistical significance of differences between the groups ($P < 0.01$).

Immunofluorescent staining and confocal laser scanning microscopy. Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min. Non-specific binding of antibodies was blocked by incubation with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. Rabbit antiserum against influenza virus A/duck/Pennsylvania/10128/84 (H5N2) or PRV was used for the detection of viral antigens. Monoclonal anti-tubulin β III isoform antibody (Chemicon International), anti-neurofilament-M&H phosphorylated forms antibody (Chemicon International) and FITC-conjugated phalloidin (Sigma-Aldrich) were used to visualize MTs, neurofilaments (NFs) and MFs, respectively. FITC-conjugated anti-mouse IgG goat serum (Cooper Biomedical) and Alexa Fluor 555-conjugated anti-rabbit IgG donkey antiserum (Molecular Probes) were used as secondary antibodies. Hoechst 33258 (Wako Pure Chemical Industries) was used for nuclear staining. Analyses were performed with an Olympus Fluoview FV500 confocal laser scanning microscope and Fluoview software version 4.2. Infectivity was scored by counting over 400 neurons on the 14 mm coverslip or over 200 neurons in the central chamber of the compartmentalized culture system.

RESULTS

Infection of cultured neuron with AIV

To evaluate the infectivity of AIV, cultured neurons were directly inoculated with the virus. At 6 h p.i., viral antigens were present mostly in the nuclei of the neurons. After that, the perikarya and axons became positive. Axons connected with infected perikarya contained dotted viral antigens on tubulin (Fig. 3a, b). A few non-neuronal cells coexisting with the neurons were also infected. The percentage of infected neurons increased gradually with incubation time (Fig. 4). No severe damage, due to infection, was observed in the neurons except for a mild swelling of cell bodies.

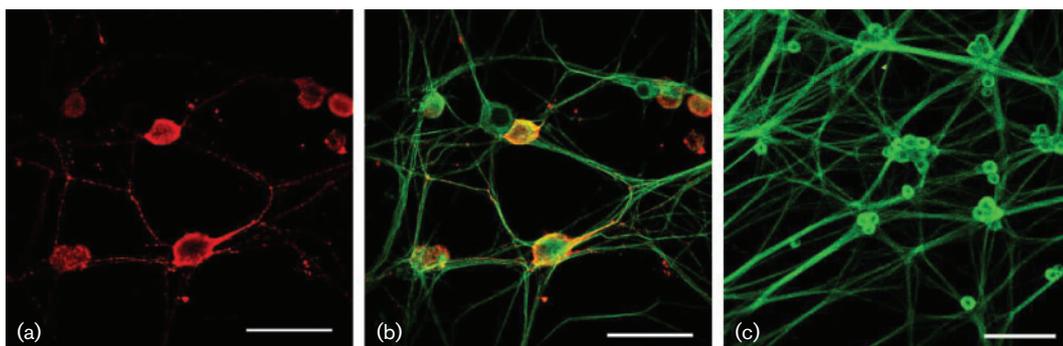


Fig. 3. Confocal images of neurons infected with influenza virus strain 24a5b and examined at 36 h p.i. (a, b) and mock-infected neurons (c). Cells were immunostained for virus antigen (a–c; red) and tubulin β III (b, c; green). (a, b) Dots of viral antigens among cell bodies are overlaid on the axons stained for tubulin. (c) Viral antigens are absent. Bars, 50 μ m (a, b); 100 μ m (c).

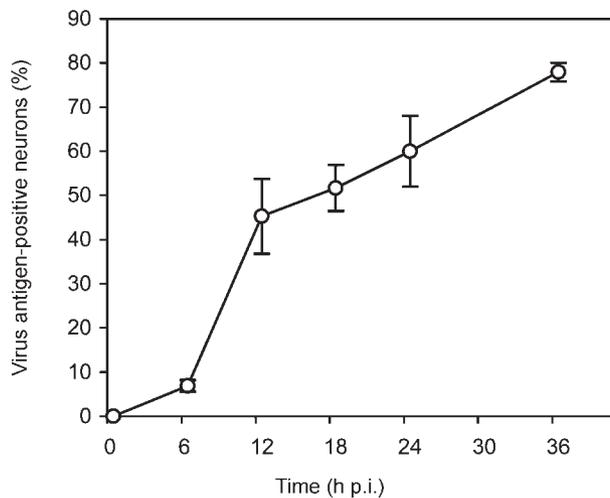


Fig. 4. Percentage of virus antigen-positive neurons after influenza virus strain 24a5b infection. Each value is expressed as the mean \pm SD.

Neurons without virus infection showed no staining for viral antigens (Fig. 3c).

Axonal transport of AIV and PRV in compartmentalized culture system

To demonstrate the axonal transport of AIV and PRV in compartmentalized neuron culture, the viruses were allowed to infect the axons in the side chambers, and then the percentage of cell bodies positive for viral antigens in the central chamber was calculated. Both viral antigens first appeared in the neuronal cell bodies at 12 h p.i., and infected neurons increased afterwards for both viruses (Fig. 5). Taking into account of the fact that only a minority of the neurons extended their axons to the side chambers (20 axons of approx. 800 surviving neurons) and that neurons in the central chamber were connected by their processes, cell-to-cell spread of the viruses among the neurons must have occurred. The pattern of infection clearly differed between the viruses: the number of cells positive for AIV increased gradually, whereas the number of cells positive for PRV increased slowly until 36 h p.i. and then abruptly from 36 to 48 h p.i. Neurons infected with PRV showed a coarsely dotted pattern of staining for viral antigens, and collapsed and unstained neurons were observed more frequently than in AIV-infected cells. Although a small number of non-neuronal cells were also infected with AIV, infected neurons were rarely associated with such non-neuronal cells.

Effects of cytoskeletal perturbation caused by drug treatment on viral spread in cultured neurons

To compare the modes of axonal transport of the two viruses, cultured neurons disrupted selectively in the

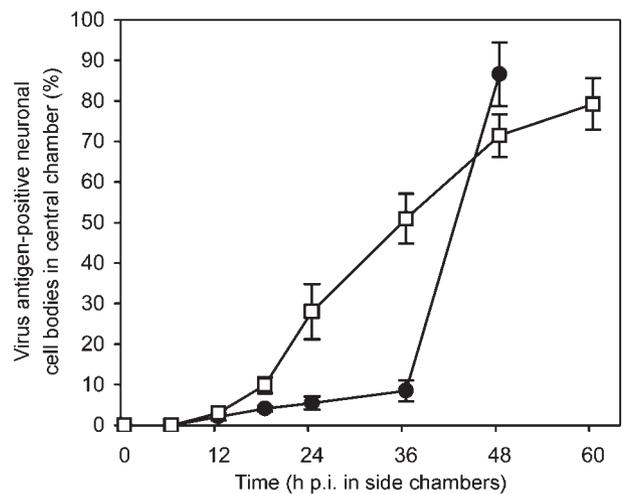


Fig. 5. Axonal transport of influenza and pseudorabies virus assayed in a compartmentalized culture system. Each value is expressed as the mean \pm SD. At each time point over 200 neurons were examined for the antigens. \square , Influenza virus strain 24a5b; \bullet , pseudorabies virus strain Yamagata S-81.

cytoskeleton, using inhibitors, were infected with the viruses. To ascertain that the adverse effects of the inhibitors on the cytoskeleton were reversible and did not kill the neurons, the infectivity of cultured neurons recovered from the drug treatment (pre-treated groups) was also compared with that of untreated control cells (schematically shown in Fig. 2). Confocal images of tubulin revealed granular to fibrillary staining in the cell bodies and fibrous staining in the untreated axons (Fig. 3b). NOC treatment strongly abated the tubulin staining and a few fine linear MTs were observed (Fig. 6b). Removal of NOC led to a re-emergence of the MTs (Fig. 6d). In the neurons treated or pre-treated with NOC, AIV spread decreased insignificantly, whereas PRV showed a 90% reduction of axonal transport in the presence of NOC (Fig. 7a). This reduction was not observed in the neurons recovered from the drug treatment. Immunofluorescent dots of both viral antigens were enlarged in the NOC-treated axons (Figs 3a, 6a and c). The morphology of actin filaments was unaffected by CYD treatment (Fig. 6e), and the percentages of antigen-positive neurons were not significantly modified by the treatment for either virus (Fig. 7b). NFs exhibited fibrillary staining in the cell bodies and axons in the untreated neurons (Fig. 6h), and neurons after recovery. With the ACR treatment, NF protein characteristically accumulated at the axon terminals and viral antigens disappeared from the axons (Fig. 6f-h). In the ACR-treated neurons, the number of cells positive for AIV decreased to 68% and that of cells positive for PRV decreased to 20% of the untreated control (Fig. 7c). These decreasing proportions of the number of neurons positive for both viruses with the ACR treatment differed significantly. The neurons recovered from ACR treatment showed a similar infectivity

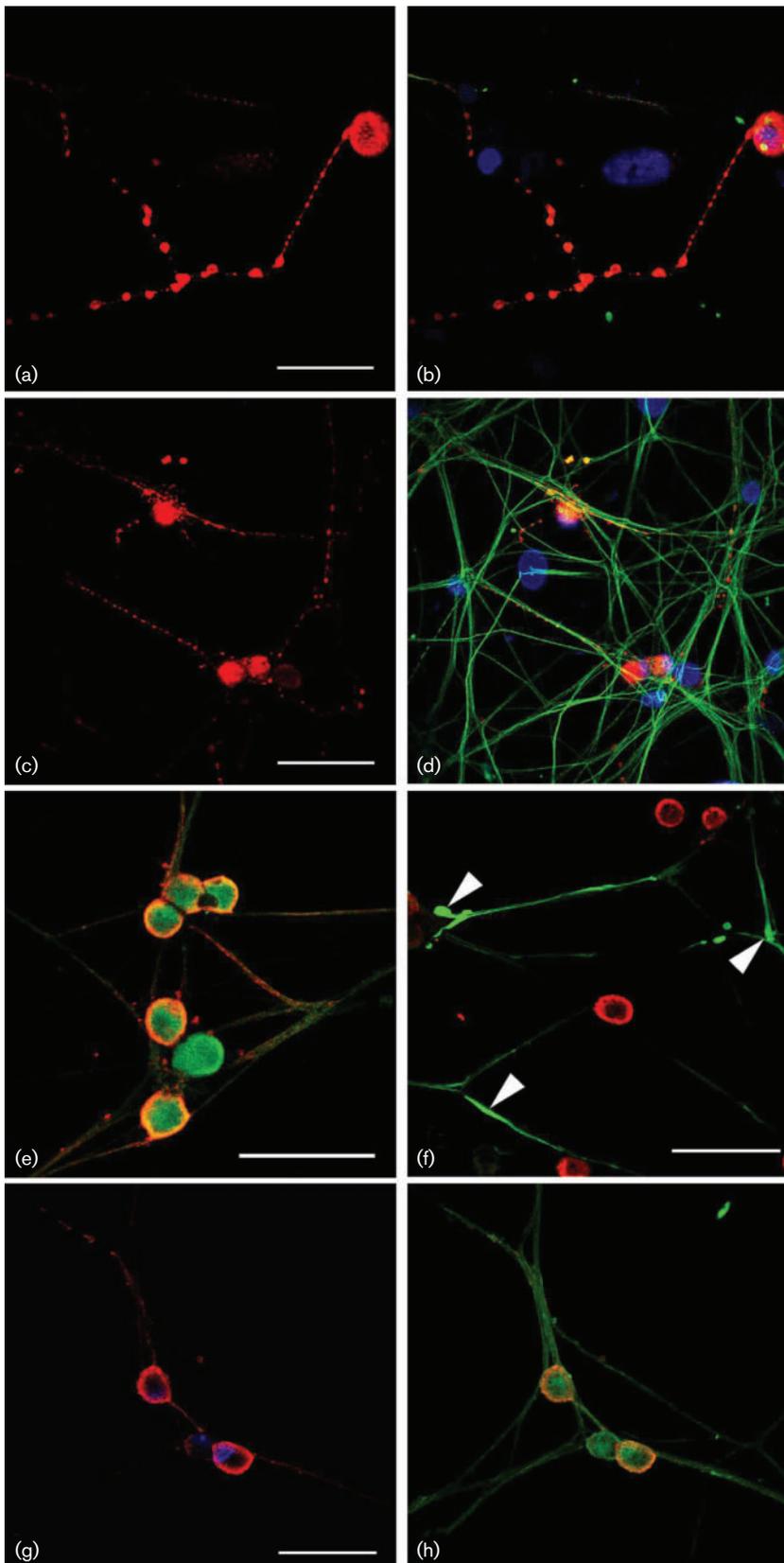


Fig. 6. Confocal images of neurons infected with influenza virus strain 24a5b with or without drug treatment. (a–d) Neurons were treated (a, b) or pre-treated (c, d) with NOC, then infected with the virus, and examined at 36 h p.i. Cells were immunostained for virus antigen (red) and tubulin β III (b, d; green). Nuclei were stained with Hoechst 33258 (b, d; blue). (a, b) Enlargement of dotted viral antigens in comparison with those in untreated neurons shown in Fig. 3(a) and (b). Microtubules almost disappeared following the NOC treatment. (c, d) Pre-treated neurons were stained for the viral antigens similarly to the untreated neurons (Fig. 3a and b), and microtubules are reconstructed. (e) Neurons were treated with CYD, infected with strain 24a5b and examined at 24 h p.i. Diffuse staining for filamentous actin is not modified by the treatment (green). Virus antigens in the perikarya and axons (red). (f–h) Neurons treated with (f) or without (g, h) ACR, infected with the virus, and examined at 24 h p.i. (f) Arrowheads indicate accumulation of neurofilament proteins at the axon terminals (green). Viral antigens are absent in the axon (red). (g, h) Fibrillary staining for neurofilament proteins throughout the cell bodies and axons. Viral antigens are within the cell bodies and axons (red). Bars, 50 μ m.

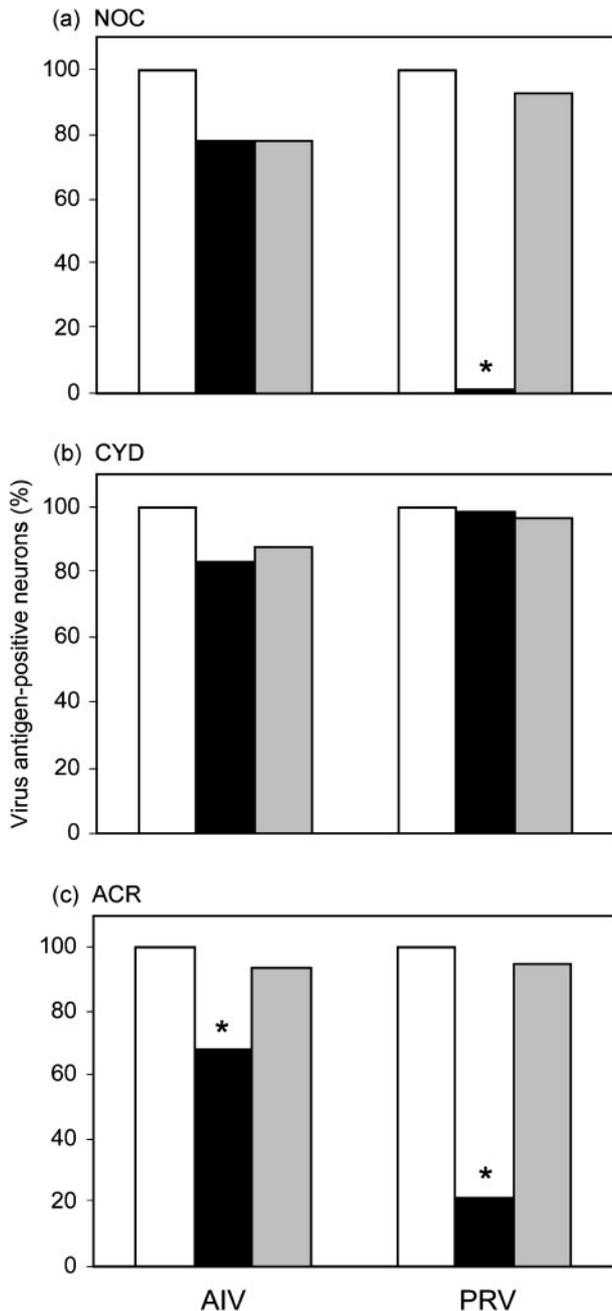


Fig. 7. Effects of cytoskeletal perturbation on viral spread in the neuronal network. Neurons were treated (black columns) or pre-treated (grey columns) with the drugs indicated on the top of each panel. Neurons were infected with influenza virus strain 24a5b (AIV) or pseudorabies virus strain Yamagata S-81 (PRV). Results are expressed as the percentage of antigen-positive cells in the drug-treated group, with the values of control groups (white columns) adjusted to 100%. In each treatment group, over 400 neurons were examined for the antigen, and the calculation was repeated more than three times using different culture dishes. Asterisk (*), statistically significant difference from untreated controls using the Student's *t*-test ($P < 0.01$).

for both viruses compared with untreated cells. The morphology of cytoskeletons was not modified by viral infection without drug treatment, and each drug treatment affected the morphology and distribution of only the target cytoskeleton.

DISCUSSION

DRG neurons do not have dendrites *in vivo*, with only a single bifurcating axon and short lateral spikes protruding from the cell body (Thomas *et al.*, 1993). Axons can be distinguished *in vivo* and *in vitro* from other cellular spikes by the presence of MTs and absence of a rough endoplasmic reticulum (Tennyson & Gershon, 1975; Yamada *et al.*, 1971). In our cultures, extensions from neuronal cell bodies were always stained by anti-tubulin β III antibody, showing them to be axons. The compartmentalized culture system has been utilized to demonstrate the axonal transport of herpes simplex virus and rabies virus (Lycke & Tsiang, 1987; Ziegler & Herman, 1980), or various biological and biochemical properties of neurons and axons (Campenot, 1977; Hayashi *et al.*, 2004; Kimpinski *et al.*, 1999). The present study with the compartmentalized culture system showed that AIV as well as PRV infecting at the distal part of axons reached neuronal cell bodies through retrograde axonal transport. In our previous papers, we have suggested transneuronal invasion of intranasally inoculated neurotropic influenza A viruses to the CNS of mice along the peripheral nerves (Matsuda *et al.*, 2004; Park *et al.*, 2002; Shinya *et al.*, 1998, 2000; Tanaka *et al.*, 2003). Several studies of influenza virus infection in brain cell cultures (Brask *et al.*, 2001; Dotti & Simons, 1990; Dotti *et al.*, 1993; Takahashi *et al.*, 1997) or in Schwann cell culture (Levine *et al.*, 2003) have been reported, but the precise mechanisms for the transneuronal spread of influenza virus, i.e. axonal and transsynaptic transport, have not been characterized. In this paper, using peripheral neurons from neonatal mouse DRG, we demonstrated for the first time the axonal transport of a neurotropic influenza A virus.

The increase in virus antigen-positive neurons in the central chamber of the compartmentalized culture system suggests that AIV as well as PRV spread transsynaptically, since the medium in the chambers contained methyl cellulose for immobilizing viruses and antiserum for neutralizing extracellular-free viruses, and that viruses spreading neuron-to-neuron via synapses are not effectively neutralized by antibodies (Price *et al.*, 1982). The machinery of the transsynaptic spread of influenza virus is unknown. Dotti *et al.* (1993) detected the haemagglutinin antigens at the axonal cell surface but failed to find budding virus from neurons infected with a fowl plague virus by immunoelectron microscopy. A possible explanation might be that the influenza viruses spread as the ribonucleoproteins encompassed in the synaptic vesicles, and that the antiserum cannot prevent the transsynaptic spread of the viruses. The rate of increase in the number of antigen-positive neurons clearly differed between AIV and PRV, and

the result suggests different modes of neuronal propagation for the two viruses, i.e. AIV was transported more promptly within the axons than PRV, and proliferated in the neurons less effectively. There is a possibility that coexisting non-neuronal cells play a substantial role for the virus spread in our culture, but, from the fact that the number of non-neuronal cells was rather small and infected neurons were rarely associated with them, we concluded that transsynaptic spread is likely to be the major cause of the infection of neurons in the central chamber.

There have been many reports concerning virus–cytoskeleton interactions, and MT and associated motor proteins, kinesin and cytoplasmic dynein, play a major role in the transport of internalized viruses to the nucleus (reviewed by Ploubidou & Way, 2001; Sodeik, 2000). However, no information is available on the mechanism of axonal transport of influenza viruses. NOC is a reversible mitotic inhibitor that binds the fast-growing ends of MTs and prevents monomer addition (Cheung & Terry, 1980; Lee *et al.*, 1980). In the present experiments, propagation of PRV was markedly inhibited by NOC treatment. This result was consistent with a previous report that PRV entered neurons by fusing their envelope with the host-cell plasma membrane and their capsid/tegument structure was transported to the nucleus on the MTs by fast axonal transport (Kaelin *et al.*, 2000; Tomishima *et al.*, 2001). The degree of propagation of PRV among the neurons recovered from the NOC treatment was equivalent to the untreated control, indicating that a reconstruction of MTs could afford the effective propagation of the virus. In contrast, the effects of MT disruption by NOC treatment were insignificant for the propagation of AIV. Lakadamyali *et al.* (2003) reported that the incoming transport of influenza A virus strain X-31 (H3N2) to the perinuclear region depends on MTs and dynein-directed motilities in Chinese hamster ovary cells. Whereas, Arcangeletti *et al.* (1997) described independence of influenza virus strain U/73 (H7N1) infection from MT construction in LLC monkey kidney epithelial cells. In the present study, AIV could spread transneurally independent of MTs, whereas the spread of PRV was sensitive to MT inhibition. These results suggest a difference in the mechanism of neuronal spread between these two viruses. However, the enlarged dots of viral antigens in MT-disrupted neurons suggest a significant, but not crucial, effect of MT on the axonal transport of AIV.

MFs and their constituent actin filaments interact with many viruses at various stages throughout their life cycles (reviewed by Cudmore *et al.*, 1997). The most extensively documented viral–actin interactions are those of vaccinia virus, which induces the formation of actin tails that launch viral particles from the cell surface on the tips of microvilli toward neighbouring cells (Cudmore *et al.*, 1995). CYD destabilizes MFs by binding to the fast-growing end of the filaments (Cooper, 1987). With the CYD treatment, the propagation of both AIV and PRV was not obviously affected. PRV has been reported not to interact with MFs

(Kaelin *et al.*, 2000; Tomishima *et al.*, 2001), which is consistent with our results. As for influenza virus infection, alternative theories for dependence on actin filaments have been reported. One study emphasized actin-dependent movements of internalized virus during the first step at the cell periphery (Lakadamyali *et al.*, 2003), and another reported that CYD-induced modifications of MFs did not significantly affect influenza virus production (Arcangeletti *et al.*, 1997). From our results, the neuronal spread of AIV was not significantly affected by CYD at the concentration we used. We chose a maximum concentration of CYD not causing detachment of neurons from the floor of the dish based on preliminary experiments. However, it is possible that actin filaments were not sufficiently disrupted at this concentration, and that actin filaments functioned normally for viral propagation.

IFs comprise diverse filamentous proteins characteristically 10–12 nm wide and their distribution is closely related to cell differentiation (reviewed by Coulombe & Wong, 2004). NFs are the major components of IFs in neurons. IFs have long been believed to simply form static ‘space filling’ cytoplasmic networks and their interactions with viruses are not well documented. Several viral infections have been shown to require intact IF networks using ACR as an IF disruption agent (Eckert, 1985), but very little is known about the roles of the IF network in viral infections (Arcangeletti *et al.*, 1997; Ashok & Atwood, 2003; Cordo & Candurra, 2003). The significant suppression of the spread of AIV and PRV in the ACR-treated cultured neurons infers that intact IF constructions are involved in the transmission of the viruses in the neuronal network. To understand the significant difference in the reduction rate of the infected neurons for both viruses by ACR, however, further experiments for virus-IF interactions are required.

Arcangeletti *et al.* (1997) attributed the dependence of influenza A virus infection on intact IFs to the potential participation of prosomes in viral protein synthesis. Prosoemes are ribonucleoprotein particles accompanying untranslated forms of mRNAs (Scherrer & Bey, 1994). Based on their close association with prosomes (Arcangeletti *et al.*, 1992; Olink-Coux *et al.*, 1992), IFs might serve as a network to guide specific prosomes, and possibly mRNAs in the cytoplasm. ACR is a water-soluble vinyl monomer and a well documented neurotoxicant in both humans and laboratory animals (reviewed by LoPachin, 2004). Axonal swelling containing an abundant NFs and degenerating mitochondria have long been considered hallmark lesions produced by the direct action of ACR at axonal or perikaryal sites. However, recent advances in the characterization of ACR neurotoxicity suggest that the nerve terminal is the primary site of ACR action and that inhibition of neurotransmission and membrane turnover in nerve terminals resulting from the ACR-induced disruption of membrane-fusion processes, contributes significantly to the pathogenesis of the neurological defects (LoPachin, 2004; LoPachin *et al.*, 2002). Based on such information, it is conceivable that the

disruption of IFs by ACR suppressed the viral spread by either preventing viral assembly and/or transmission by IFs, inducing prosome dysfunction or interfering with membrane fusion at synapses.

In conclusion, we demonstrated here, for the first time *in vitro*, the axonal transport of avian influenza virus and MT-independent neuronal propagation of the influenza virus.

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