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Studies on Infection and Propagation of Neurotropic Viruses in Cultured Dorsal Root Ganglia Cells

神経親和性ウイルスの培養背根神経節細胞における感染性と増殖性の研究

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List of Abbreviations

ACR	acrylamide
CNS	central nervous system
CYD	cytochalasin D
DRG	dorsal root ganglia
FFU	focus forming unit
HEV	swine hemagglutinating encephalomyelitis virus 67N strain
MM	maintenance medium
NOC	nocodazole
p.i.	post inoculation
PRV	pseudorabies virus strain Yamagata-S 81
RV	rabies virus CVS strain
TCID ₅₀	50% tissue culture infectious dose

General Introduction

Many viruses which invade the central nervous system (CNS) via the peripheral nervous system (neural spread) have been reported ever since and they induced serious damage of central and peripheral nervous system to both animal and human. The studies on the infection and propagation mechanisms of these viruses are useful to understand the characters of them and finally they may be related to the development of therapeutic or preventive measures.

Sensory neurons are most probably involved in the virus transfer from the site of the primary infection to the CNS. Dorsal root ganglia (DRG) are crucial in the initial events or the ground of secondary viral propagation of the *in vivo* and *in vitro* infection by neurotropic viruses, since they can act as gates for the virus entrance into the CNS (Coulon *et al.*, 1989; Hirano *et al.*, 2004; Jackson and Reimer, 1989; Park *et al.*, 2006). DRG has been useful in the understanding of these initial virus-host interaction events. In the previous report, Matsuda *et al.* (2005) demonstrated that neural spread of pseudorabies virus (PRV), a neurotropic alphaherpes virus, in cultured nerve cells obtained from DRG of newborn mice required the integrity of microtubules and intermediate filaments of the cells, whereas neural spread of influenza A virus was independent of microtubule integrity.

In this study, I chose three neurotropic viruses; swine hemagglutinating encephalomyelitis virus (HEV), rabies virus (RV) and PRV, which show neural spread in animals. HEV belongs to the order *Nidovirales*, family

Coronaviridae, genus *Coronavirus*. The virus was first isolated in Canada from the brains of suckling piglets (Pensaert, 2006), and identified as causes of encephalomyelitis or vomiting and wasting disease in suckling piglets (Cutlip and Mengeling, 1972; Pensaert, 2006). In previous *in vivo* studies using piglets and mice, HEV has been suggested to invade into the CNS by neural spread (Andries and Pensaert, 1980; Hirano *et al.*, 2004; Pensaert, 2006; Yagami *et al.*, 1986; Yagami *et al.*, 1993). PRV, also known as Aujeszky's disease virus or suid herpes virus 1, belongs to the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* and it has been demonstrated to propagate transaxonally *in vivo* and *in vitro* studies (Card *et al.*, 1990; Enquist *et al.*, 2002; Field and Hill, 1974). RV belongs to the family *Rhabdoviridae*, genus *Lyssavirus*. RV is highly neurotropic and migrates to the neuronal soma by retrograde axonal transport and finally released into the CNS, resulting in lethal encephalitis in various animals (Jackson, 2006; Jackson, 2007; Murphy, 1977).

This thesis is composed of two chapters. In chapter 1, I compared neurotropism and the role of cytoskeletal components in the neural spread of HEV with those of PRV using cultured nerve cells and non-neuronal cells from DRG of newborn mice. In chapter 2, I examined cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral nucleoprotein using cultured nerve cells and non-neuronal cells from DRG of newborn mice.

Chapter 1

Comparative studies on infection and propagation between swine hemagglutinating encephalomyelitis virus and pseudorabies virus in the primary cultured mouse dorsal root ganglia cells

Introduction

Swine hemagglutinating encephalomyelitis virus (HEV) belongs to the order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*. The virus was first isolated in Canada from the brains of suckling piglets (Pensaert, 2006), and causes encephalomyelitis or vomiting and wasting disease in suckling piglets (Cutlip and Mengeling, 1972; Pensaert, 2006). In previous *in vivo* studies using piglets and mice, HEV has been suggested to invade the CNS via the peripheral nervous system (neural spread) (Andries and Pensaert, 1980; Hirano *et al.*, 2004; Pensaert, 2006; Yagami *et al.*, 1986; Yagami *et al.*, 1993). However, the neural spread of HEV has not been examined by *in vitro* studies.

Matsuda *et al.* (2005) demonstrated that the neural spread of pseudorabies virus (PRV), a neurotropic alphaherpesvirus, in cultured nerve cells obtained from dorsal root ganglia (DRG) of newborn mice required the integrity of microtubules and intermediate filaments of the cells, whereas

the neural spread of influenza A was independent of microtubule integrity. In this chapter, I compared neurotropism of HEV with that of PRV. The roles of neuronal cytoskeletal components in the neural spread of HEV was also compared with those of PRV using cultured nerve cells and non-neuronal cells from DRG of newborn mice.

Materials and Methods

Viruses

The HEV 67N strain was first isolated from the nasal cavities of apparently healthy feeder pigs in 1972 in the United States. This virus was propagated in FS-L3 cells (Sasaki *et al.*, 2003). 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) was the first Japanese isolate from piglets (Itakura *et al.*, 1981). This virus was propagated in cloned porcine kidney (CPK) cells.

DRG cell culture

DRG of the spinal cords of newborn ICR/Jcl mice (2-4 days old) were dissociated by incubation with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis,

MO, U.S.A.) at 37°C for 30 min. Dissociated cells were resuspended at a concentration of 50,000 nerve cells (about 100 ganglia) per ml in maintenance medium (MM) comprised of Eagle's minimal essential medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD, U.S.A.), 50 U/ml penicillin (GIBCO, Gaithersburg, NY, U.S.A.) and 50 µg/ml streptomycin (GIBCO). Collagen-coated 35 mm dishes with central 14 mm glass coverslips (Matsunami Glass, Osaka, Japan) 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, Carlsbad, CA, U.S.A.) in a 5% CO₂ atmosphere at 37°C. The medium was changed every 2-3 days. After 7-10 days of incubation, when an axonal network of nerve cells had formed, dishes were used for the experiments. In each dish, non-neuronal cells were admixed with nerve cells. All the experiments using experimental animals were conducted in accordance with the policies of the Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University.

Infection of cultured DRG cells with HEV and PRV

Cultured DRG cells on the 14 mm coverslip were inoculated with either HEV at 10^{6.7} TCID₅₀/ml or PRV at 10^{4.37} TCID₅₀/ml in 100 µl of MM and incubated for 1 hr at 37°C. The viral suspension was removed and cells were washed three times with MM, then incubated in MM for 24, 48 and 60 hr. At

least three samples were examined for each time point. Mock-infected DRG cells were used as a negative control.

Cytoskeletal interference and viral propagation in cultured DRG cells

Nocodazole (NOC; Sigma-Aldrich) was used for the disruption of microtubules, acrylamide (ACR; Sigma-Aldrich) for the perturbation of intermediate filaments and cytochalasin D (CYD; Sigma-Aldrich) for the disruption of microfilaments. Full-grown nerve cells with axonal networks were prepared on 14 mm coverslips in 35 mm dishes. The cultured DRG cells were treated with NOC at 1, 5 and 10 μ M or ACR at 1, 2 and 3 mM for 23 hr before infection. In the case of CYD, DRG cells were treated at 1, 2.5 and 5 μ M for 2 hr before infection. After that the cells were inoculated with either HEV at $10^{6.7}$ TCID₅₀/ml or PRV at $10^{4.37}$ TCID₅₀/ml in 100 μ l of MM and incubated for 1 hr at 37°C. The inoculum was then removed by washing with MM three times and MM was applied with each drug. At 2 hr after inoculation with each virus, the inoculum of CYD was then removed by washing with MM three times and MM was applied without CYD and incubated until 48 hr after inoculation with each virus. At 48 hr after inoculation with each virus, infected DRG cells in all dishes were fixed for morphological examination. Stock solutions of NOC (10 mM) and CYD (1 mM) in DMSO and ACR (1 M) in double-distilled water were prepared and diluted to the final concentration in MM. The maximum concentrations of

the inhibitors were chosen to maximize the drug effects without any apparent cytotoxic effect on cell morphology. The intermediate and minimum concentrations were used to confirm the effect of inhibitors. Controls were treated with an equal volume of DMSO or double-distilled water. 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 ($P < 0.01$ and $P < 0.05$). Each experiment was performed at least three times.

Immunocytochemical analysis

DRG cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min. Nonspecific binding of antibodies was blocked by incubation with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. Newly prepared mouse antiserum against HEV and rabbit antiserum against PRV (a gift from Dr. E. Ono) was used for the detection of viral antigens. A mouse monoclonal anti-tubulin β III isoform antibody (Chemicon International, Temecula, CA, U.S.A.), rabbit polyclonal anti-tubulin β III isoform antibody (Abcam, Cambridge, UK), mouse anti-neurofilament-M&H phosphorylated form antibody (Chemicon International), rabbit polyclonal anti-pan neurofilament antibody (BIOMOL International, Plymouth Meeting, PA, U.S.A.) and Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, U.S.A.) were used to visualize microtubules, neurofilaments

and microfilaments, respectively. Alexa Fluor 488-conjugated anti-rabbit IgG donkey serum (Molecular Probes), Alexa Fluor 488-conjugated anti-mouse IgG donkey serum (Molecular Probes), Alexa Fluor 555-conjugated anti-mouse IgG donkey serum (Molecular Probes) and Alexa Fluor 555-conjugated anti-rabbit IgG donkey serum (Molecular Probes) were used as secondary antibodies. Hoechst 33258 (Wako Pure Chemical Industries, Osaka, Japan) was used for nuclear staining. Analyses were performed with an Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan) and Fluoview software version 5.0 (Olympus). For calculating the infectivity of each virus, images of 5 fields were viewed at random, and the number of antigen-positive DRG cells was counted. 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. Each experiment was repeated at least three times.

Results

Infectivity of viruses in nerve cells in cultured DRG

The percentage of antigen-positive nerve cells in the 14 mm coverslip was counted at each time point. Nerve cells were identified by immunohistochemistry using the anti-tubulin β III antibody in our cultured DRG cells. The time courses of propagation of both viruses in DRG nerve

cells were similar and cells that were antigen-positive for both viruses increased time dependently. In HEV infection, viral antigen-positive nerve cells were not detected at 0 and 24 hr post inoculation (p.i.) (Fig. 1A, B), but after that they sharply increased. At 48 hr p.i. (Fig. 1C), about 85% of the nerve cells were viral antigen-positive and this figure reached 90% at 60 hr p.i. (Fig. 1D). The viral antigens of HEV first appeared at the cell periphery of nerve cells and then extended to the whole cytoplasm of the cells. After that, the viral antigens were detected in the axons (Fig. 1C, D). In PRV infection, viral antigen-positive nerve cells were not detected at 0 hr p.i. (Fig. 1E) and slowly increased until 24 hr p.i. (Fig. 1F). After that, the number of positive cells sharply increased from 24 to 48 hr p.i. At 48 hr p.i., 100% of the cells were viral antigen-positive (Fig. 1G, 2). The viral antigens appeared first in nuclei or perinuclear regions (Fig. 1F), and then moved to periphery of the neuronal cell bodies (Fig. 1G). After that, viral antigen-positive granules appeared in the axons (Fig. 1H). No severe damage due to virus infection was observed in the nerve cells except for mild swelling of cell bodies (Fig. 1).

Infectivity of viruses in non-neuronal cells in cultured DRG

In this cultured DRG cells, non-neuronal cells coexisted with nerve cells. The non-neuronal cells were not stained by the anti-tubulin β III antibody. These cells were spindle-shaped with fusiform nuclei or irregularly shaped

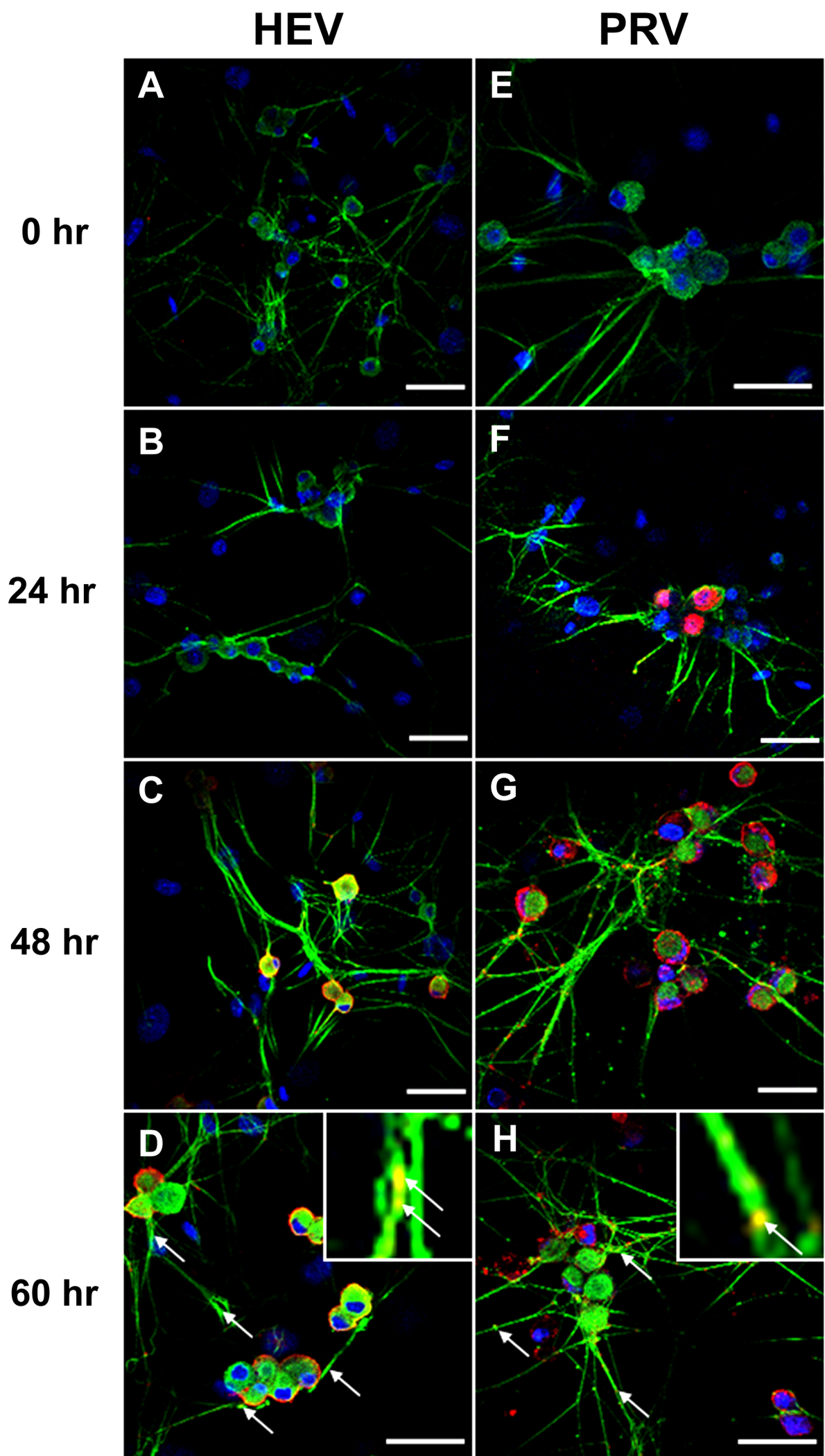
with flattened nuclei. The non-neuronal cells were not infected with HEV but 34% of them became PRV antigen-positive at 48 hr p.i. (Fig. 3, 4).

Effects of cytoskeletal perturbation on viral propagation

The cytoskeletal morphology was not modified by viral infection without drug treatment, and each inhibitor specifically affected the morphology of the targeted cytoskeletal component. The nerve cells untreated with NOC had confocal images of granular to fibrillary tubulin staining (Fig. 5A, E). Nerve cells treated with NOC lost these characteristics depending on the concentration of the inhibitor (Fig. 5B, C, F, G), and only a few fine linear microtubules were observed at the maximum concentration (Fig. 5D, H). The number of PRV-positive cells was reduced drastically with an increase of the NOC concentration in the medium (Fig. 5F, G) and PRV-positive nerve cells were scarcely observed in dishes treated with 10 μ M NOC (Fig. 5H). In contrast, the number of HEV-positive nerve cells gradually decreased with the increase of the NOC concentration (Fig. 5B-D, 8A). Neurofilaments (intermediate filaments) exhibited fibrillary staining in control nerve cells (Fig. 6A, E). With ACR treatment, neurofilament protein characteristically accumulated at the axon terminals and this change became evident with the increased ACR concentrations (Fig. 6B-D, F-H). Cells that were antigen-positive for HEV or PRV decreased with increased ACR concentrations (Fig. 6, 8B). The morphological changes of actin filaments

(microfilaments) were few by CYD treatment, and the percentages of antigen-positive nerve cells were not significantly different from the control for both viruses (Fig. 7, 8C).

Fig. 1. Confocal images of cultured nerve cells after HEV and PRV infection. The cultured nerve cells are immunostained for viral antigens (red), tubulin β III (green) and nuclei (blue). Viral antigens are HEV (A-D) and PRV (E-H). Confocal images of cultured nerve cells at 0 hr p.i. (A, E), at 24 hr p.i. (B, F), at 48 hr p.i. (C, G) and at 60 hr p.i. (D, H). Insets in D and H are higher magnification of axons. Arrows indicate viral antigens in the axons (yellow). Bars=100 μ m.



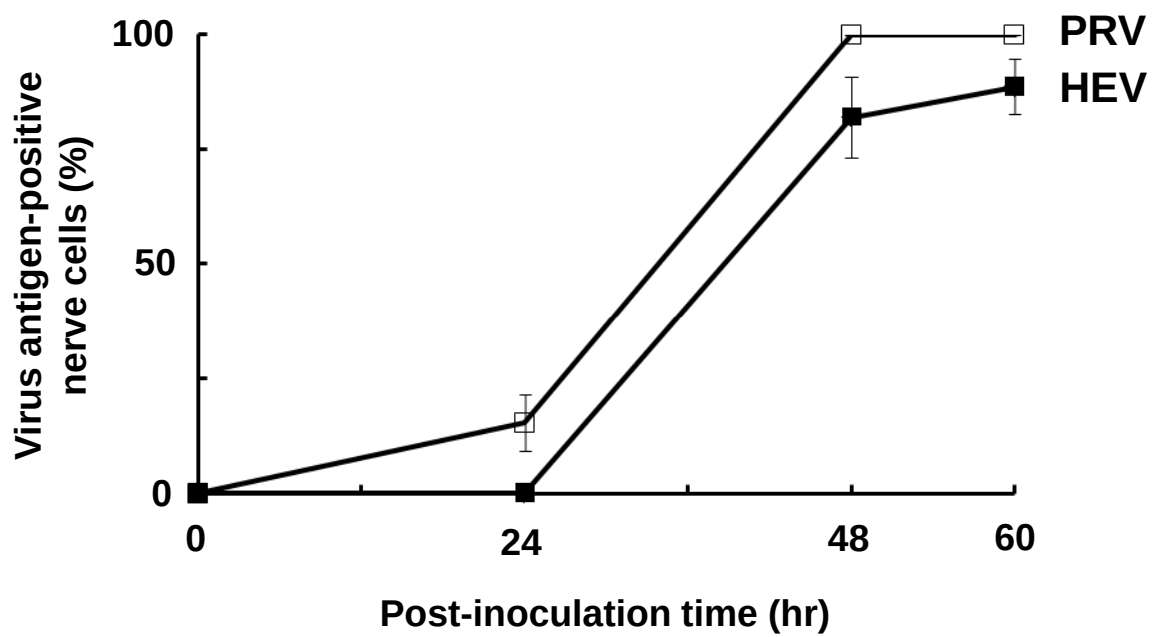
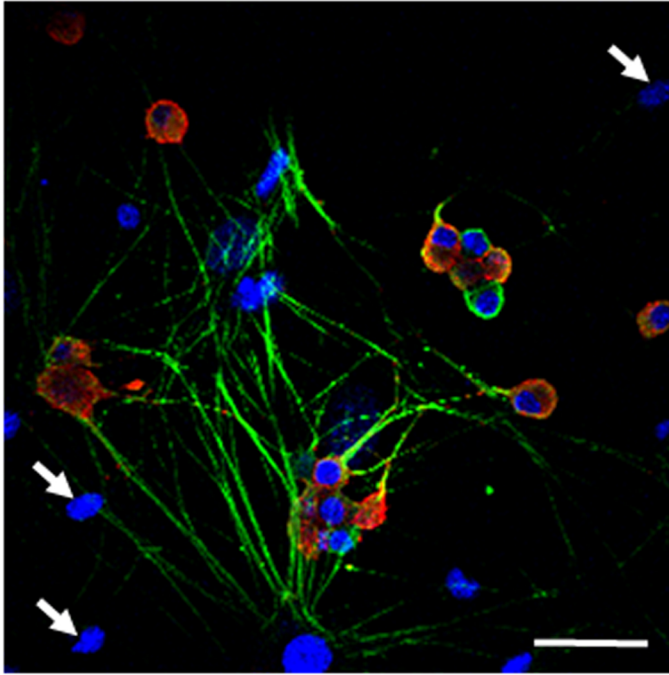
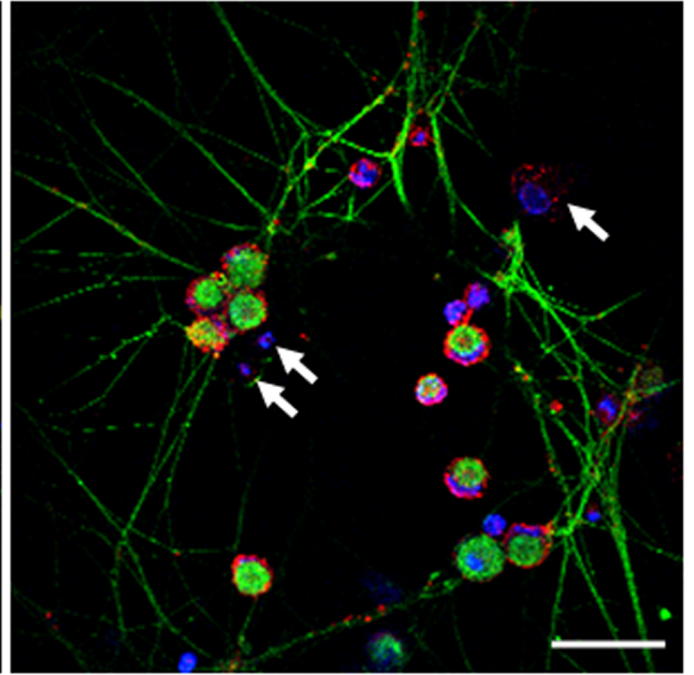


Fig. 2. Percentages of virus antigen-positive nerve cells in cultured DRG cells. Closed squares are HEV and open squares are PRV. Each value is expressed as the mean $\pm$ SD.

Fig. 3. Confocal images of virus antigen-positive non-neuronal cells in cultured DRG cells infected with HEV and PRV. The cultured DRG cells are immunostained for viral antigens (red), tubulin β III (green) and nuclei (blue). Nerve cells are immunostained for tubulin β III and non-neuronal cells are not stained for tubulin β III. Arrows indicate non-neuronal cells. Bars=100 μ m.



HEV



PRV

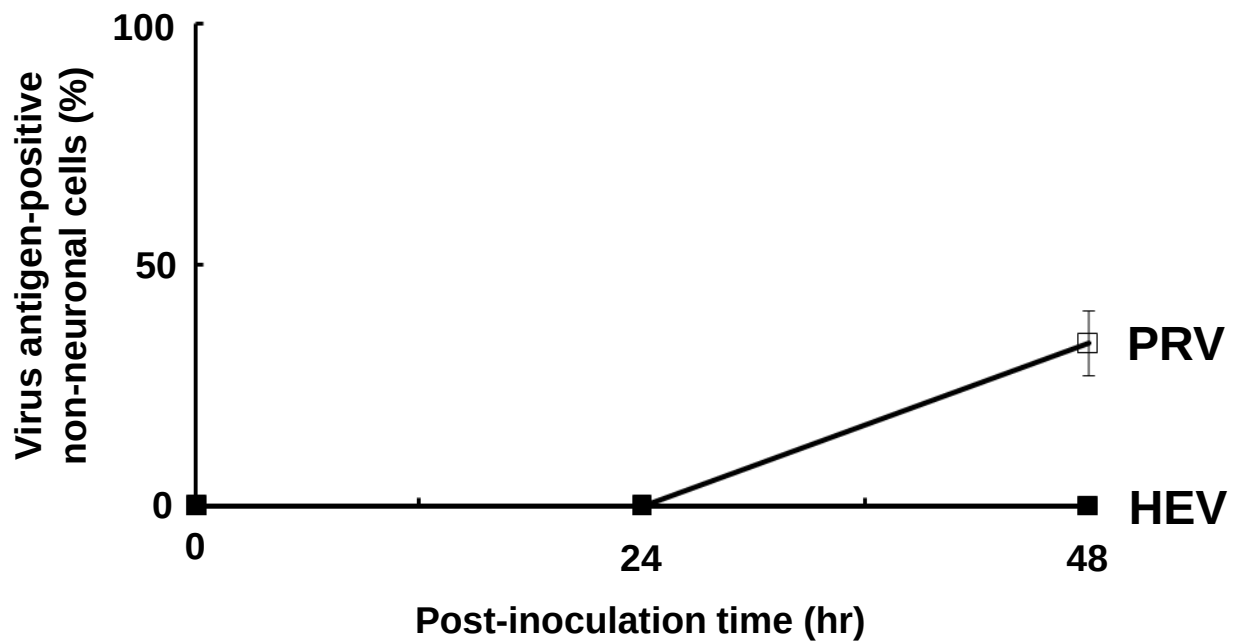


Fig. 4. Percentages of virus antigen-positive non-neuronal cells in cultured DRG cells. Closed squares are HEV and open squares are PRV. Each value is expressed as the mean $\pm$ SD.

Fig. 5. Confocal images of virus antigen-positive nerve cells with NOC treatment. Confocal images of nerve cells are immunostained for viral antigens (red), tubulin β III (green) and nuclei (blue). Nerve cells were infected with HEV (A-D) or PRV (E-H). Concentrations of NOC are 1 μ M (B, F), 5 μ M (C, G) and 10 μ M (D, H). As for control, vehicle of NOC (A, E) was used. Bars=100 μ m.

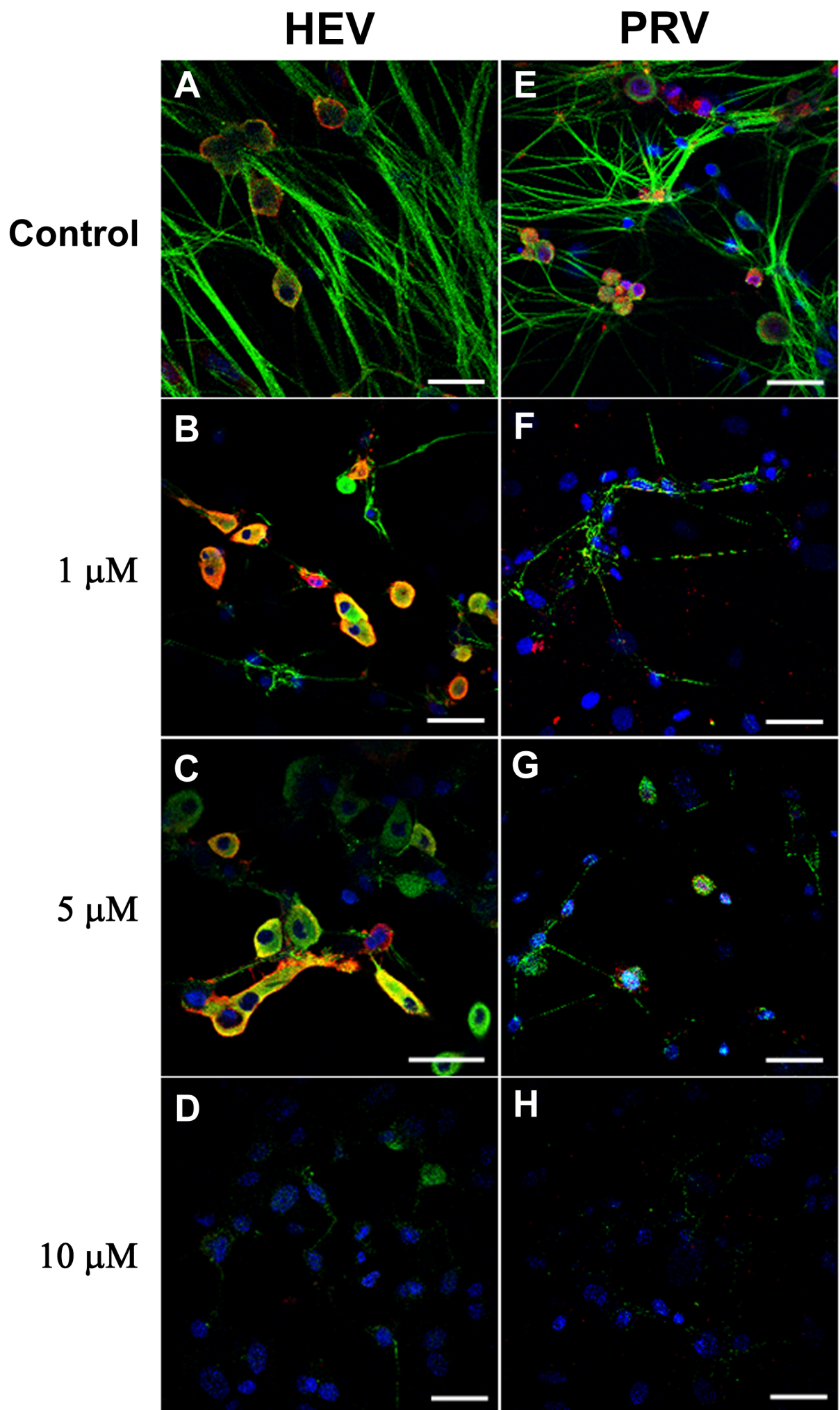


Fig. 6. Confocal images of virus antigen-positive nerve cells with ACR treatment. Confocal images of nerve cells are immunostained for viral antigens (red), neurofilaments (green) and nuclei (blue). Nerve cells were infected with HEV (A-D) or PRV (E-H). Concentrations of ACR are 1 mM (B, F), 2 mM (C, G) and 3 mM (D, H). As for control, vehicle of ACR (A, E) was used. Bars=100 μ m.

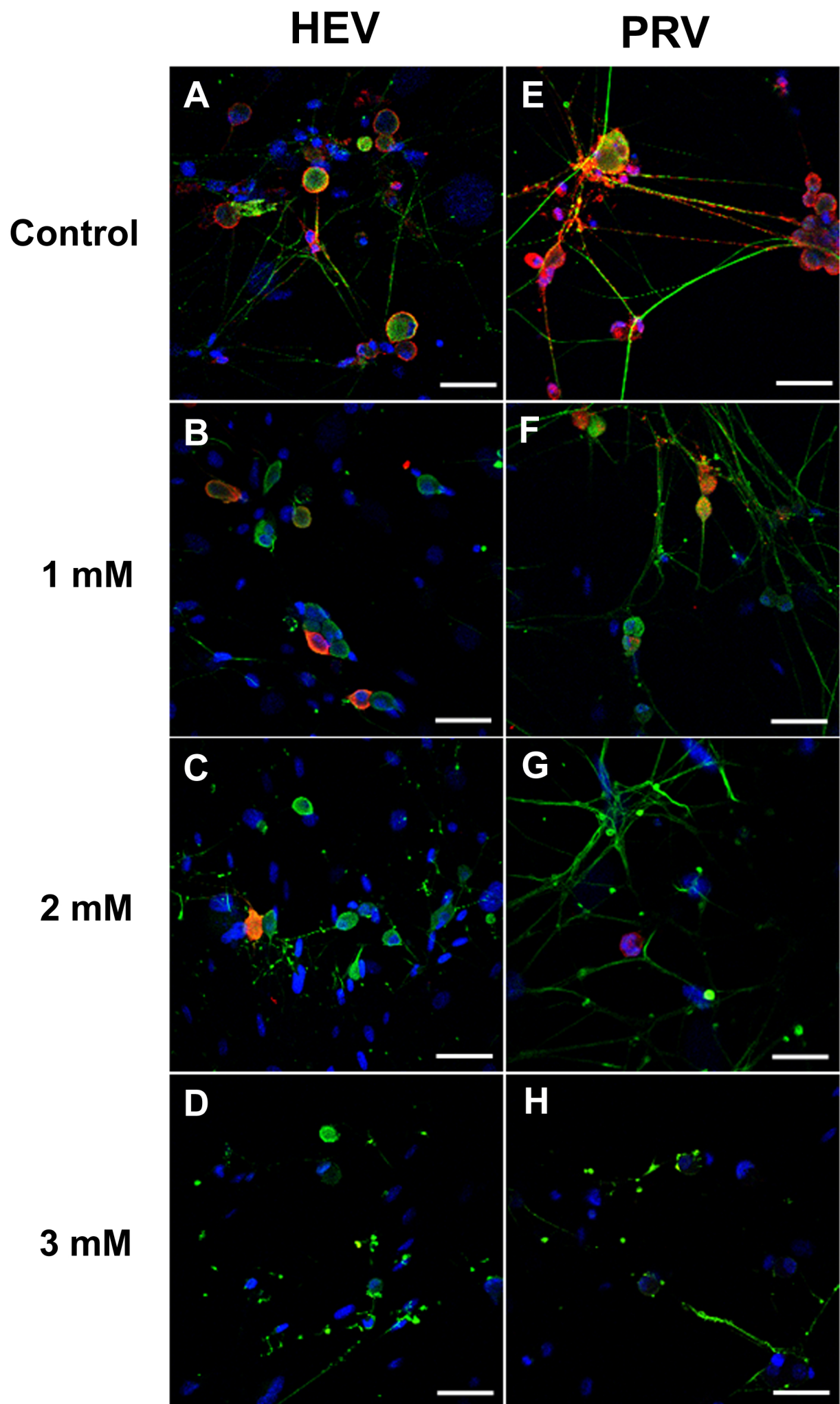


Fig. 7. Confocal images of virus antigen-positive nerve cells with CYD treatment. Confocal images of nerve cells are immunostained for viral antigens (red), actin filaments (green) and nuclei (blue). Nerve cells were infected with HEV (A-D) or PRV (E-H). Concentrations of CYD are 1 μ M (B, F), 2.5 μ M (C, G) and 5 μ M (D, H). As for control, vehicle of CYD (A, E) was used. Bars=100 μ m.

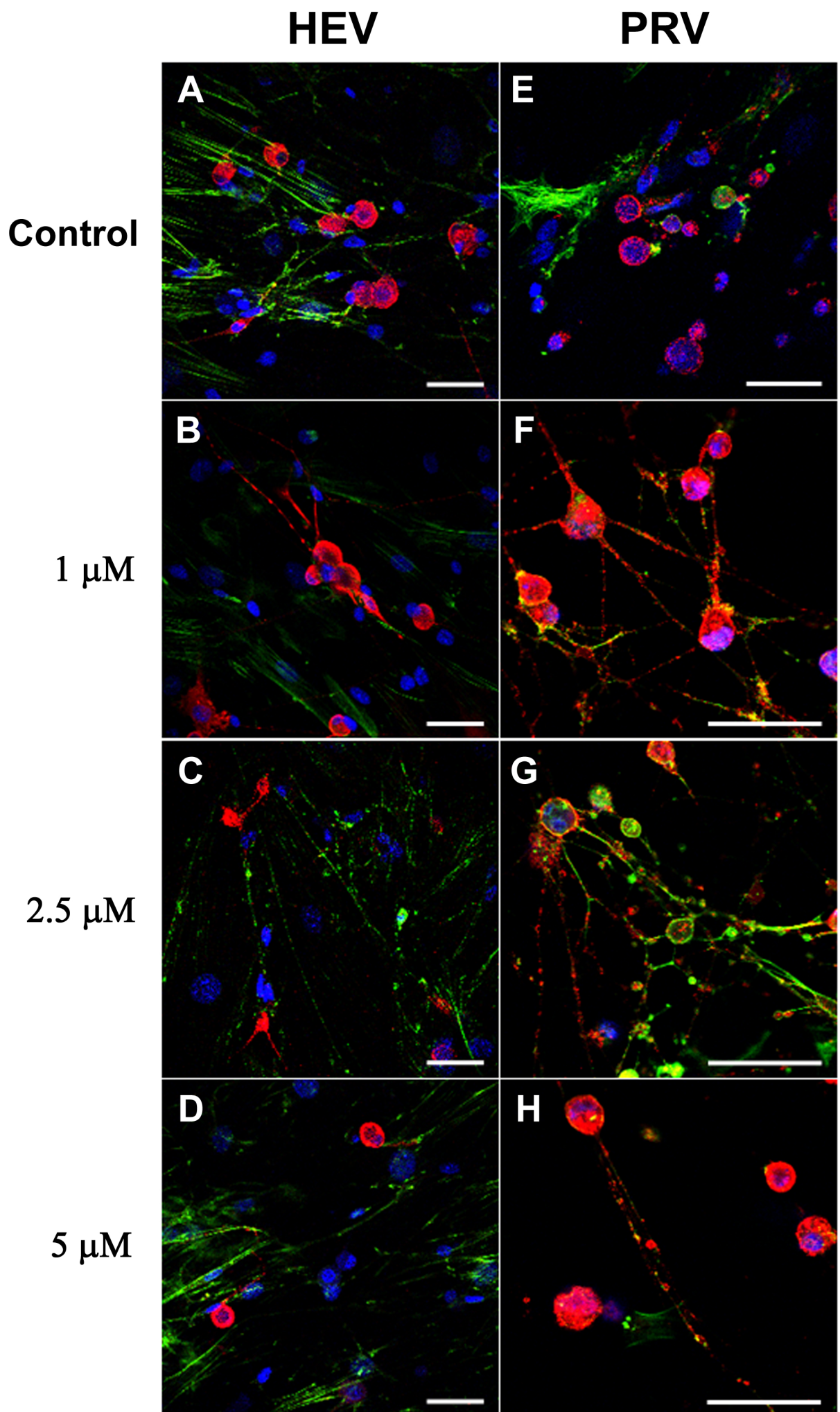
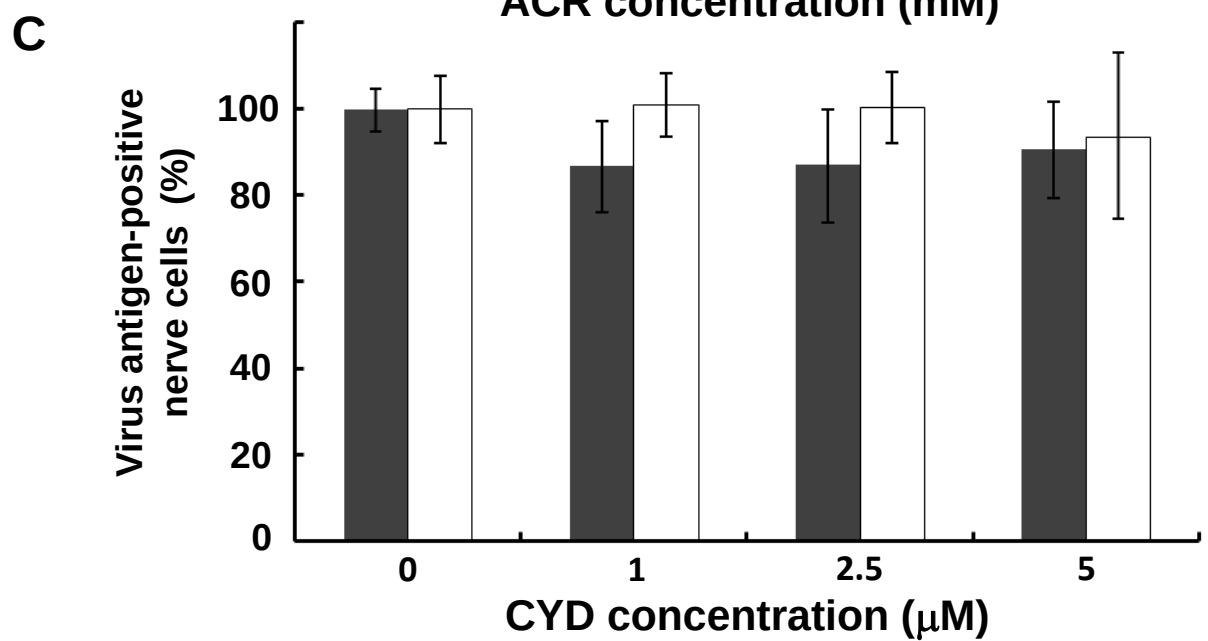
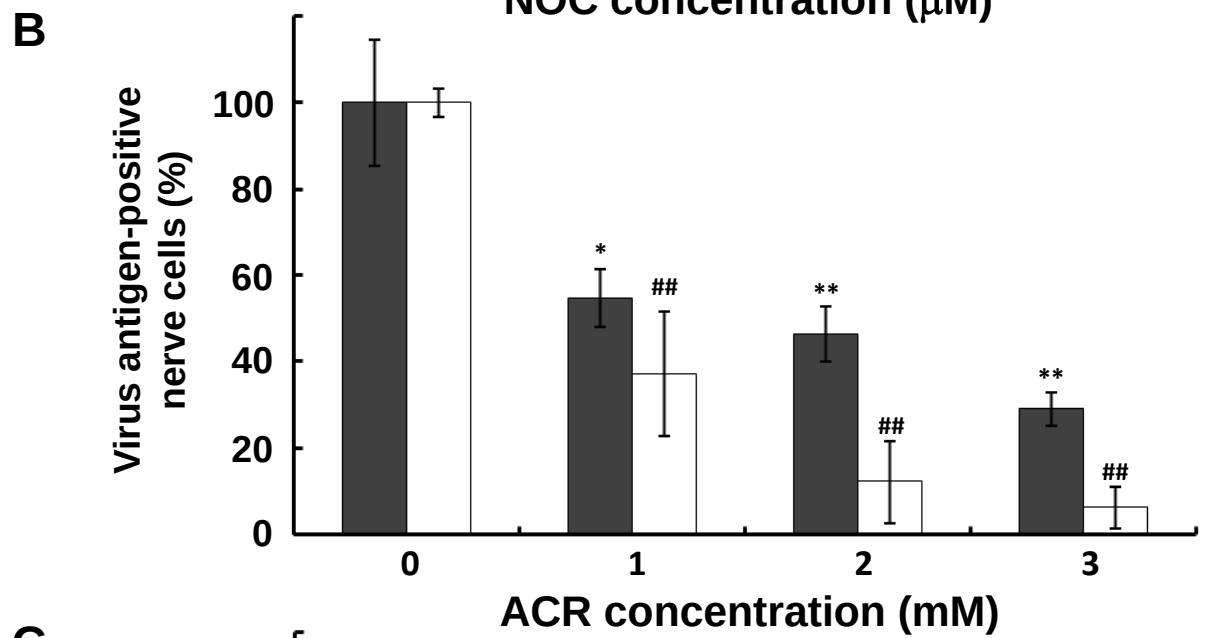
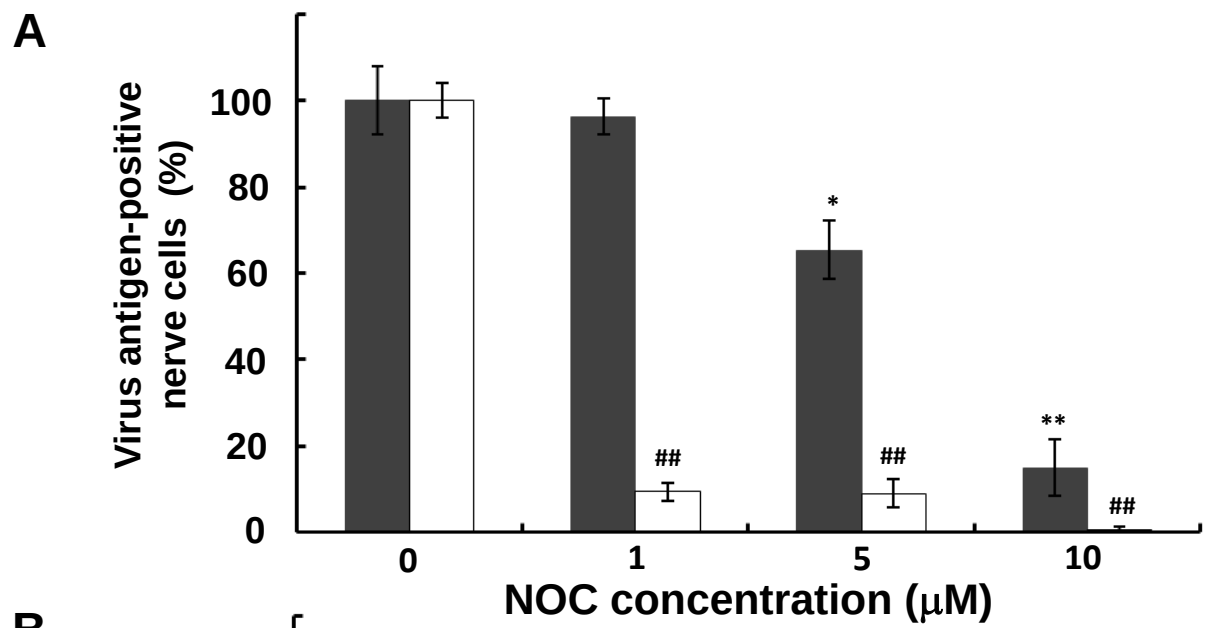


Fig. 8. Effects of cytoskeletal perturbation on viral infection in cultured nerve cells treated with the cytoskeletal inhibitors indicated at the bottom of each panel. Nerve cells were infected with HEV (black columns) or PRV (white columns). Results are expressed as the percentage of viral antigen-positive nerve cells for each concentration of the cytoskeletal inhibitors. Values of the control group are adjusted to 100%. Columns and vertical bars show means $\pm$ SD. ** $p < 0.01$, ## $p < 0.01$ and * $p < 0.05$ vs. controls.



Discussion

The cultured newborn mouse DRG cells used in this study were composed of nerve cells and non-neuronal cells. These findings were consistent with previous studies on cultured mouse DRG cells (Andersen *et al.*, 2003; Enquist *et al.*, 2002; Fleming *et al.*, 2001; Sommer *et al.*, 1985; Tennyson and Gershon, 1975). Nerve cells were identified by their characteristic spherical cell bodies, fine axonal projections, small spherical nuclei, and immunohistochemical staining with the neuron-specific tubulin β III antibody. Non-neuronal cells were composed of two kinds of cells: spindle-shaped cells with fusiform nuclei and irregularly shaped cells with flattened nuclei. Based on the morphology of these non-neuronal cells, the former were considered to be Schwann cells and the latter fibroblasts (Andersen *et al.*, 2003; Fleming *et al.*, 2001). In the present study, PRV infected nerve cells and non-neuronal cells, but HEV antigen was found only in nerve cells. These *in vitro* findings were consistent with previous *in vivo* studies in which PRV antigens were found in astrocytes and nerve cells (Card *et al.*, 1990; Field and Hill, 1974), whereas HEV particles could be found in nerve cells but were not detected in glial cells and infiltrating cells (Hirano *et al.*, 2001; Hirano *et al.*, 2004; Meyvisch and Hoorens, 1978). Based on these *in vivo* and *in vitro* findings, infectivity of HEV may be more strictly restricted to nerve cells than PRV and rabies virus, for which non-neuronal cells may support viral entry and replication (Lycke and Tsiang, 1987).

In general, coronaviruses bind to specific cell surface receptors via the

surface projectional glycoproteins and then replicative cycles occur in the cytoplasm (Macnaughton, 1987; Mengeling *et al.*, 1972; Mengeling and Cutlip, 1972; Meyvisch and Hoorens, 1978). Complete virions assemble to the rough endoplasmic reticulum or Golgi apparatus (Koga and Ushiki, 2006; Macnaughton, 1987; Sommer *et al.*, 1985). In a previous electron microscopic study on HEV encephalitis in piglets, viral particles were observed in the cytoplasm of infected neurons; most of the particles were found close to the cytoplasmic membrane and some were confined to the endoplasmic reticulum or Golgi structures (Macnaughton, 1987). In this study, HEV antigens localized to the periphery of nerve cells in the early stage of infection and then spread to the entire cytoplasm and axon terminals. These results are consistent with the general lifecycle of coronaviruses in the infected cells.

To understand the relation between HEV infection and neuronal cytoskeletal components (microtubules, intermediate filaments, and microfilaments), cultured nerve cells disrupted selectively for these components with specific inhibitors were exposed to HEV and PRV. NOC is a reversible mitotic inhibitor that binds the fast-growing ends of microtubules and prevents monomer addition (Cheung and Terry, 1980; Lee *et al.*, 1980). ACR is a water-soluble vinyl monomer and has been used as an intermediate filament disruption agent (Eckert, 1985). ACR inhibits neurotransmission and membrane turnover in nerve terminals (LoPachin, 2004; LoPachin *et al.*, 2002). CYD is a small, naturally occurring organic molecule and destabilizes microfilaments by binding to the fast-growing end of the filaments (Cooper,

1987). In this study, viral infection of PRV in nerve cells was inhibited by NOC and ACR treatment in a dose-dependent manner by the inhibitors, whereas CYD treatment did not affect the infectivity of the viruses. These results for PRV were consistent with previous reports (Kaelin *et al.*, 2000; Matsuda *et al.*, 2005; Tomishima *et al.*, 2001) and suggest that PRV depends on microtubules of nerve cells to transport its capsid-tegument structure to the nucleus using fast axonal flow (Kaelin *et al.*, 2000; Ploubidou and Way, 2001; Sodeik, 2000; Tomishima *et al.*, 2001). The intermediate filament disruption by ACR suggested that infection and propagation of PRV depend on intermediate filaments of nerve cells for viral assembly and/or membrane fusion at synapses (Matsuda *et al.*, 2005). PRV has been reported not to interact with microfilaments (Kaelin *et al.*, 2000; Tomishima *et al.*, 2001). I used maximally 5 μ M CYD in the present study but the disruption of microfilaments in the nerve cells was not morphologically apparent. This phenomenon was same as previous report (Matsuda *et al.*, 2005). It is difficult to discuss the role of microfilaments in the infection and propagation of both PRV and HEV. Interactions of HEV with the cellular cytoskeleton have not been reported, but previous reports on the localization of viral antigens in HEV-infected neurons suggest that HEV uses microtubules and intermediate filaments in transporting viral endocytic vesicles to the Golgi apparatus and/or rough endoplasmic reticulum (Cheung and Terry, 1980; Lee *et al.*, 1980), and in viral assembly and/or membrane fusion at synapses. Although the magnitude of the inhibitory effects on viral infection was less prominent in HEV, disruption of the cytoskeletal components by inhibitors

similarly affected the percentage of HEV and PRV infection in the present study. Therefore, the mode of viral infection and propagation within and among nerve cells may be similar for HEV and PRV, and the two viruses may be transported between the cell body and axon terminals of neurons by fast axonal flow. But the mode of the process was distinctive from that of influenza A virus (Matsuda *et al.*, 2005).

Summary

HEV causes encephalomyelitis or vomiting and wasting disease in suckling piglets. Neurotropism of the virus has been demonstrated in previous *in vivo* studies. In the present study, I investigated the infectivity of HEV in comparison with those of PRV, another neurotropic virus, using DRG cells of newborn mice containing nerve cells and non-neuronal cells. HEV infected nerve cells but did not infect non-neuronal cells, whereas PRV infected both cell types. By using cytoskeletal inhibitors, it was suggested that infection and propagation of HEV and PRV within and among nerve cells depended on microtubules and intermediate filaments of nerve cells, indicating that the viruses may be associated with fast axonal flow to be transported between the cell body and axonal terminals of neurons.

Chapter 2

Infection and propagation of rabies virus in the primary cultured mouse dorsal root ganglia cells

Introduction

Rabies virus (RV) belongs to the family *Rhabdoviridae*, genus *Lyssavirus*. Rhabdoviruses are enveloped with a typical bullet-or rod-shaped morphology and are characterized by an extremely broad host spectrum ranging from plants to insects and mammals (Jackson, 2006; Jackson, 2007). RV is highly neurotropic and migrates to the neuronal soma by retrograde axonal transport from the periphery, and then is taken by anterograde axonal transport to be finally released into the CNS, resulting in lethal encephalitis (Jackson, 2006; Jackson, 2007; Murphy, 1977). Under natural conditions, humans and animals may experience long and variable incubation periods following bite exposure. This may play a role in maintaining enzootic rabies, especially in high-density, high-contact populations where there is a tendency for the disease to burn itself out by rapidly reducing the number of susceptible animals. In humans, the incubation period is usually between 20 and 90 days, although incubation periods may be as short as a few days or longer than a year (Jackson, 2006; Jackson, 2007).

Peripheral nerves of both motor and sensory neurons can be used for propagation of rabies virus, although sensory neurons are most probably involved in the transfer of the virus from the site of the primary infection to the CNS (Jackson and Reimer, 1989; Lycke and Tsiang, 1987; Tsiang and Atanasiu, 1975; Tsiang *et al.*, 1991). Fibers from neurons of DRG are crucial in the initial events of the infection by the rabies virus since they can act as gates for the viral entrance into the CNS (Coulon *et al.*, 1989; Jackson and Reimer, 1989; Park *et al.*, 2006). In addition, they have been useful in understanding initial virus-host interactions.

In the previous report, Matsuda *et al.* (2005) demonstrated that neural spread of PRV in cultured nerve cells obtained from DRG of newborn mice required the integrity of microtubules and intermediate filaments of the cells, whereas neural spread of influenza A virus was independent of microtubule integrity. In this chapter, I examined the cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral nucleoprotein (N protein) using cultured nerve cells and non-neuronal cells from DRG of newborn mice.

Materials and Methods

Rabies Virus

The CVS strain (a gift from Dr. N. Minamoto, Gifu University) was used in this experiment. The strain was propagated in C1300 clone NA mouse neuroblastoma (NA) cells. The virus was titrated in NA cells by indirect immunofluorescence assay using a monoclonal antibody against rabies virus N protein clone 6-4 (a gift from Dr. N. Minamoto).

DRG cell culture

The materials and methods of DRG cell culture was same as described in chapter 1.

Infection of cultured DRG cells with RV

Cultured DRG cells on the 14 mm coverslip were inoculated with RV at 6.3×10^6 FFU/ml or 6.3×10^5 FFU/ml in 100 μ l of MM (126 MOI or 12.6 MOI, respectively) and incubated for 1 hr at 37°C. The viral suspension was removed and cells were washed three times with MM, then incubated in MM for 24, 48 and 72 hr. At least three samples were examined for each time point. Mock-infected DRG cells were used as a negative control.

Cytoskeletal interference and viral propagation in cultured DRG cells

NOC (Sigma-Aldrich) was used for the disruption of microtubules, ACR (Sigma-Aldrich) for the perturbation of intermediate filaments and CYD (Sigma-Aldrich) for the disruption of microfilaments. Fully grown nerve cells with axonal networks were prepared on 14 mm coverslips in 35 mm dishes. The cultured DRG cells were treated with NOC at 1, 5 and 10 μ M or ACR at 1, 2 and 3 mM for 23 hr before the infection. In the case of CYD, DRG cells were treated at 1, 2.5 and 5 μ M for 2 hr before infection. After that, the cells were inoculated with RV at 6.3×10^6 FFU/ml in 100 μ l of MM and incubated for 1 hr at 37°C. The inoculum was then removed by washing with MM three times and MM was applied with each drug. At 2 hr after inoculation with RV, the inoculum of CYD was removed by washing with MM three times and MM was applied without CYD and incubated until 48 hr after inoculation with RV. At 48 hr after inoculation with RV, infected DRG cells in all dishes were fixed for morphological examination. Stock solutions of NOC (10 mM) and CYD (1 mM) in DMSO and ACR (1 M) in double-distilled water were prepared and diluted to the final concentration in MM. The maximum concentrations of the inhibitors were chosen to maximize the drug effects without any apparent cytotoxic effect on cell morphology. Intermediate and minimum concentrations were used to confirm the effects of inhibitors. Controls were treated with an equal volume of DMSO or double-distilled water. 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 ($P < 0.01$). Each experiment was performed at least three times.

Immunocytochemical analysis

DRG cells were fixed with 4% paraformaldehyde (Merck) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min. Nonspecific binding of antibodies was blocked by the incubation with 2% bovine serum albumin (Sigma-Aldrich) for 30 min. A mouse monoclonal antibody against rabies virus N protein clone 6-4 (a gift from Dr. N. Minamoto) was used for the detection of viral antigens. A rabbit polyclonal anti-tubulin β III isoform antibody (Abcam), rabbit polyclonal anti-pan neurofilament antibody (BIOMOL International) and Alexa Fluor 488-conjugated phalloidin (Molecular Probes) were used to visualize microtubules, neurofilaments and microfilaments, respectively. Alexa Fluor 488-conjugated anti-rabbit IgG donkey serum (Molecular Probes) and Alexa Fluor 555-conjugated anti-mouse IgG donkey serum (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 (Olympus) and Fluoview software version 5.0 (Olympus). For calculating the infectivity of RV, images of 5 fields were viewed at random, and the number of antigen-positive DRG cells was counted. 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. Each experiment was repeated at least three times.

Results

Infectivity of RV in nerve cells in cultured DRG

To evaluate the infectivity of RV in newborn mouse DRG nerve cells, the percentage of antigen-positive nerve cells in the 14 mm coverslip was counted at 0, 24, 48, 72 hr post inoculation (p.i.). Nerve cells were identified by immunohistochemistry using the anti-tubulin β III antibody in our cultured DRG cells. Viral antigen-positive nerve cells were not detected at 0 hr p.i. in DRG cells infected with 6.3×10^6 FFU/ml of RV (Fig. 9A). At 24 hr p.i., the viral antigens were localized in perikarya of nerve cells (Fig. 9B). At 48 hr p.i., viral antigen was observed in small round accumulations in the cytoplasm (Fig. 9C). From 24 to 72 hr p.i., the number of viral antigen-positive nerve cells was slightly increased in a time dependent manner (Fig. 10). However, the maximum percentage of antigen-positive nerve cells was about 45% at 72 hr p.i. (Fig. 9D, 10). In DRG cells infected with 6.3×10^5 FFU/ml of RV, the number of the viral antigen-positive nerve cells was also gradually increased in a time dependent manner and about 40% of nerve cells were antigen-positive at 72 hr p.i. (Fig. 10). No severe damage due to virus infection was observed in nerve cells except for mild swelling of cell bodies (Fig. 9). Mock-infected nerve cells showed no staining for viral antigens.

Infectivity of RV in non-neuronal cells in cultured DRG

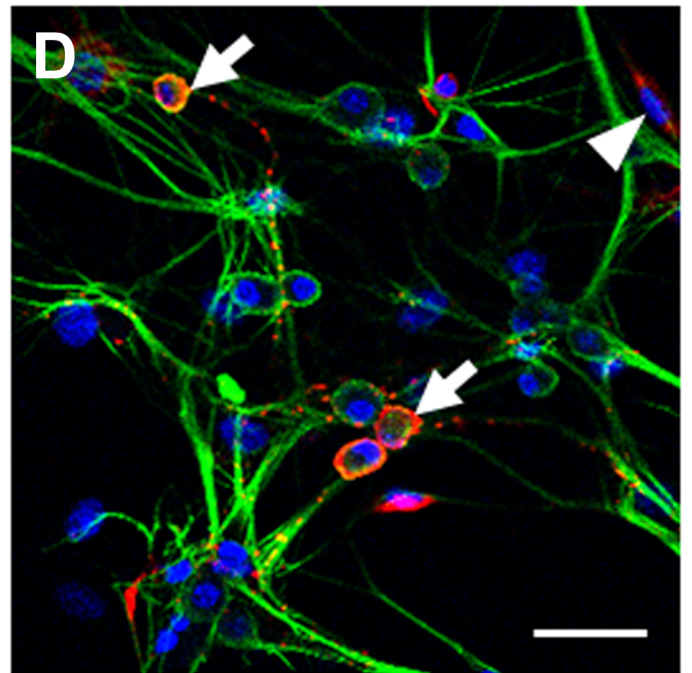
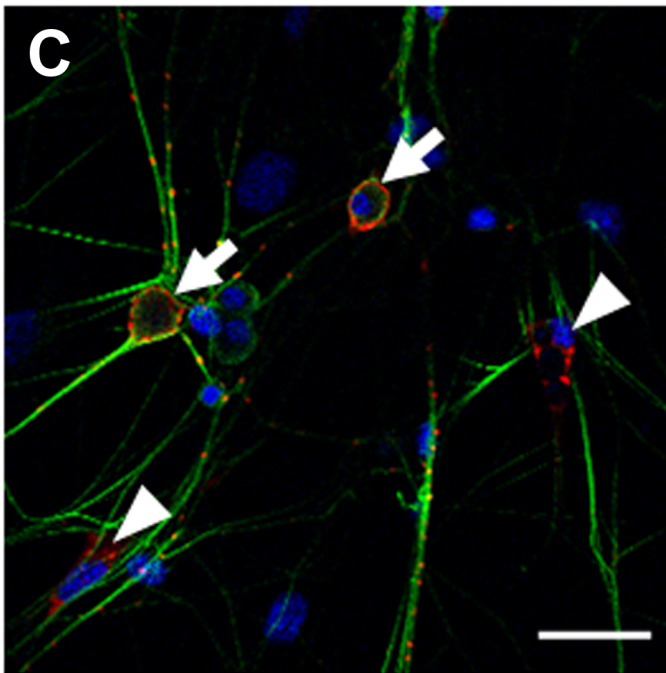
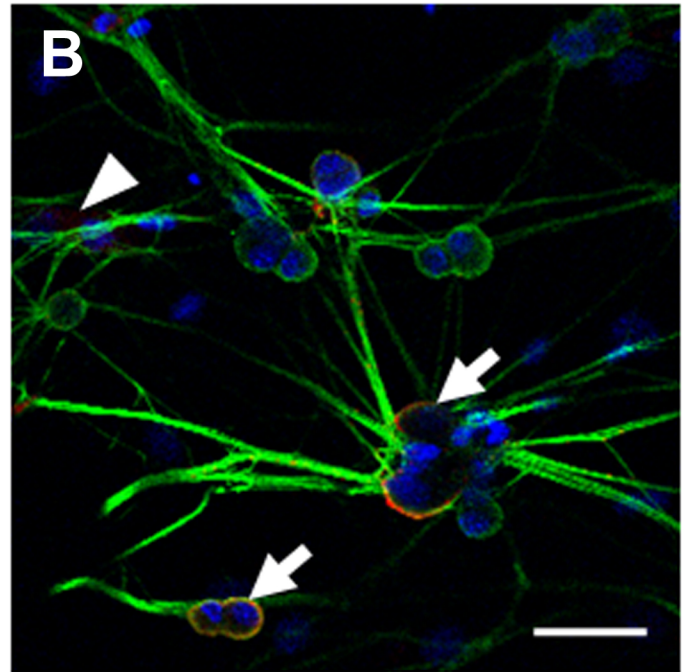
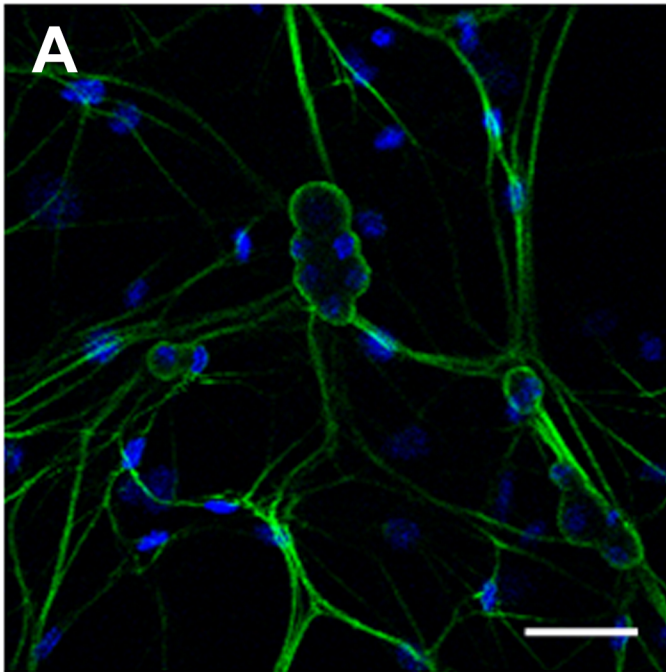
In this cultured DRG cells, non-neuronal cells coexisted with nerve cells. The non-neuronal cells were not stained with the anti-tubulin β III antibody. The non-neuronal cells were spindle-shaped with fusiform nuclei or irregularly shaped with large flattened nuclei. The non-neuronal cells that coexisted with the nerve cells were also infected with RV (Fig. 9). The percentages of viral antigen-positive cells were increased with time. At 72 hr p.i., those of antigen-positive non-neuronal cells reached about 30% of all non-neuronal cells at 6.3×10^6 FFU/ml inoculation and about 20% at 6.3×10^5 FFU/ml inoculation (Fig. 11).

Effects of cytoskeletal perturbation on viral N protein synthesis

The untreated nerve cells had confocal images of granular to fibrillary tubulin, neurofilaments and microfilaments staining (Fig. 12A, 13A, 14A). Nerve cells treated with NOC lost normal tubulin characteristics depending on the concentration of the inhibitor (Fig. 12B, C), and only a few fine linear microtubules were observed at the maximum concentration (Fig. 12D). The number of viral antigen-positive cells increased significantly at the maximum concentration but the increase did not show dose dependency at the low concentrations (Fig. 15A). With ACR treatment, neurofilament protein characteristically accumulated at the axon terminals and this change became evident with increased ACR concentrations (Fig. 13B-D). The percentage of antigen-positive nerve cells was not significantly different from

the control (Fig. 15B). The morphological changes of the cells treated with CYD were few, but the percentage of antigen-positive nerve cells was decreased (Fig. 14B-D). There is no statistical difference from the control (Fig. 15C).

Fig. 9. Confocal images of cultured DRG cells after RV infection at 6.3×10^6 FFU/ml in 100 μ l MM. The DRG cells are immunostained for viral antigen (red), tubulin β III (green) and nuclei (blue). Confocal images of cultured DRG cells at 0 hr p.i. (A), at 24 hr p.i. (B), at 48 hr p.i. (C) and at 72 hr p.i. (D). Nerve cells are immunostained by tubulin β III and non-neuronal cells are not stained by tubulin β III. Arrows indicate nerve cells and arrowheads indicate non-neuronal cells. Bars=100 μ m.



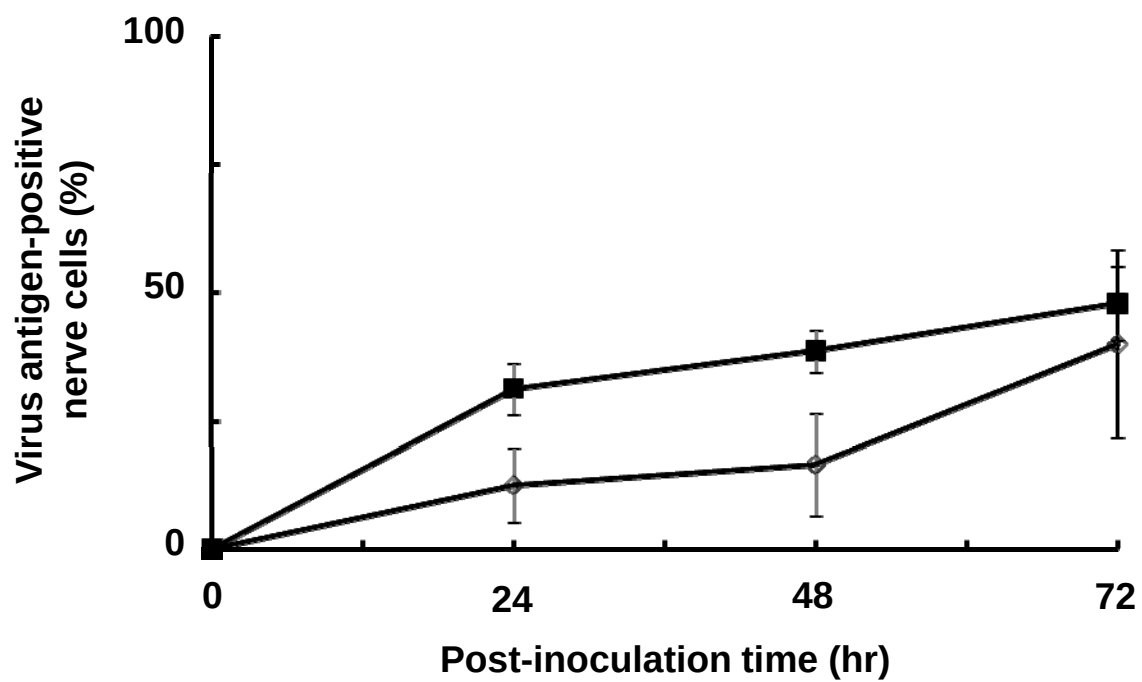


Fig. 10. Percentages of virus antigen-positive nerve cells after RV infection. Closed squares represent antigen-positive nerve cells infected with 6.3×10^6 FFU/ml of RV and open diamonds those cells infected with 6.3×10^5 FFU/ml. Each values expressed mean $\pm$ SD.

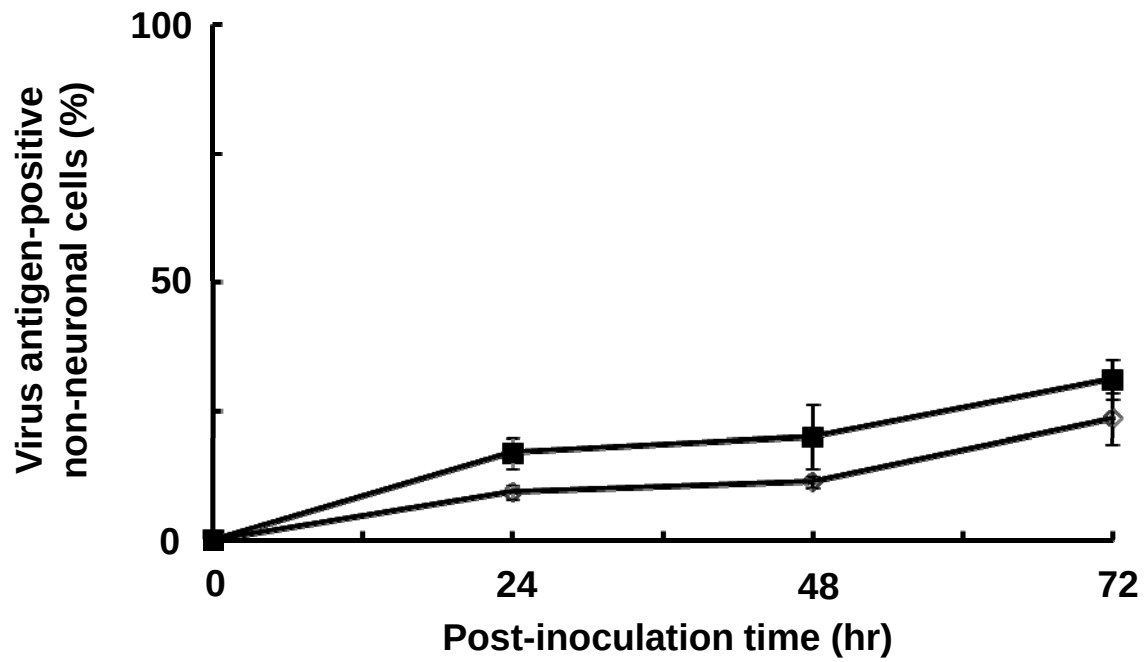


Fig. 11. Percentages of virus antigen-positive non-neuronal cells after RV infection. Closed squares represent antigen-positive non-neuronal cells infected with 6.3×10^6 FFU/ml of RV and open diamonds those cells infected with 6.3×10^5 FFU/ml. Each value is expressed as mean $\pm$ SD.

Fig. 12. Confocal images of RV antigen-positive nerve cells with NOC treatment. Confocal images of nerve cells are immunostained for viral antigen (red), tubulin β III (green) and nuclei (blue). Nerve cells were infected with 6.3×10^6 FFU/ml of RV. Concentrations of NOC are 1 μ M (B), 5 μ M (C) and 10 μ M (D). As for control, vehicle of NOC (A) was used. Bars=100 μ m.

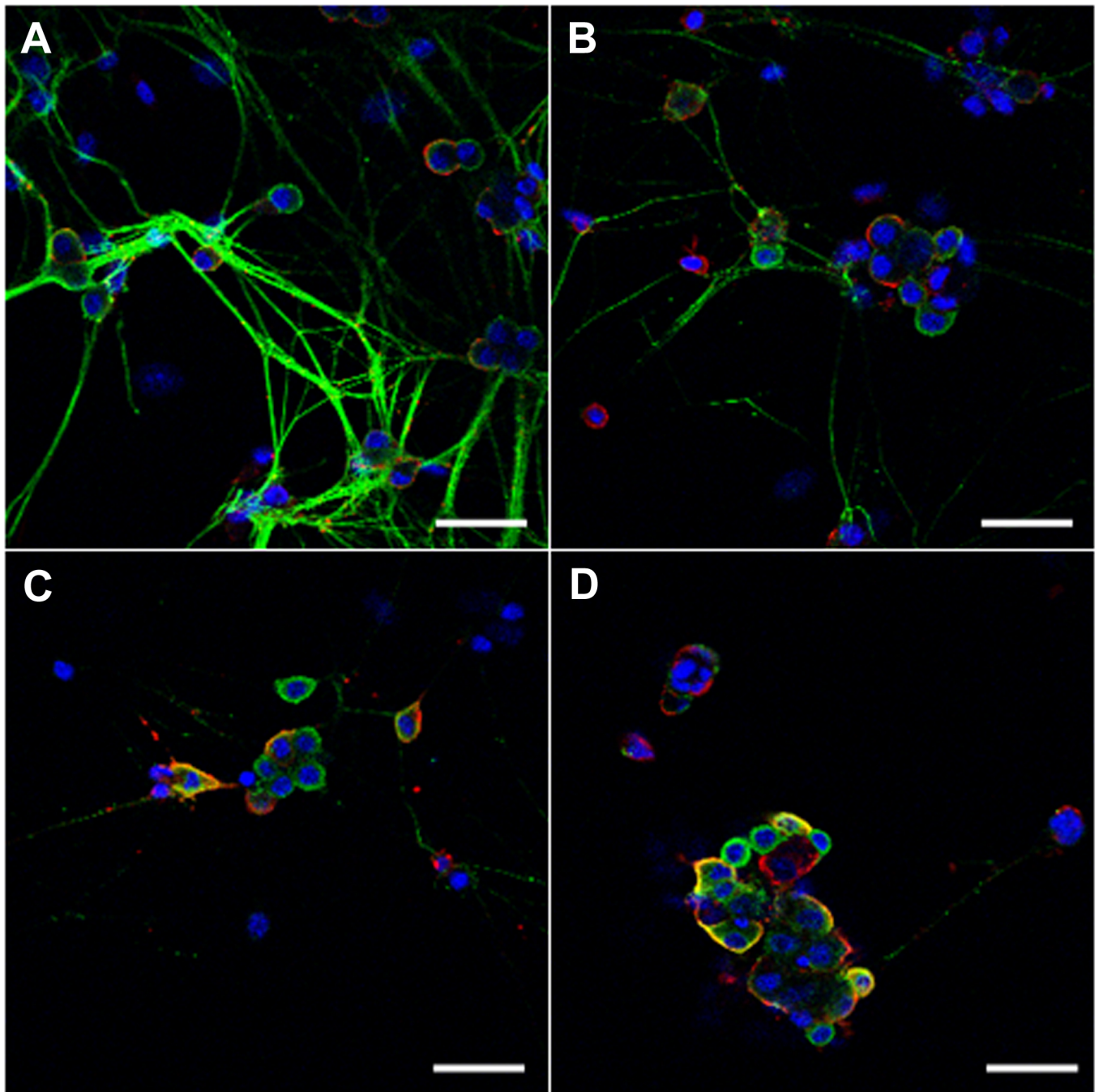


Fig. 13. Confocal images of RV antigen-positive nerve cells with ACR treatment. Confocal images of nerve cells are immunostained for viral antigen (red), neurofilaments (green) and nuclei (blue). Nerve cells were infected with 6.3×10^6 FFU/ml of RV. Concentrations of ACR are 1 mM (B), 2 mM (C) and 3 mM (D). As for control, vehicle of CYD (A) was used. Bars=100 μm .

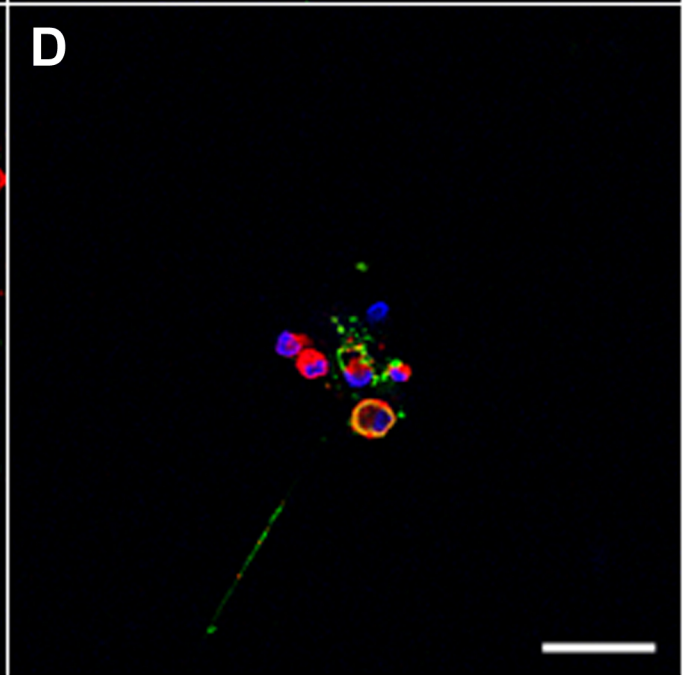
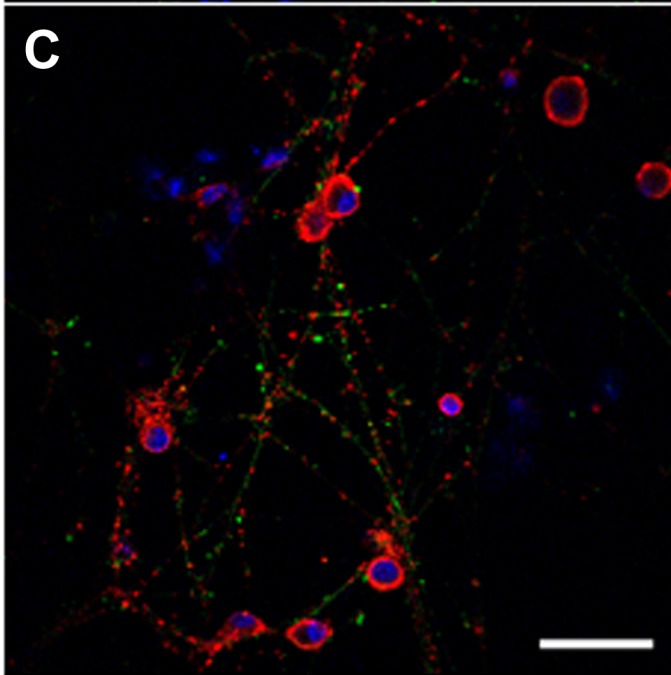
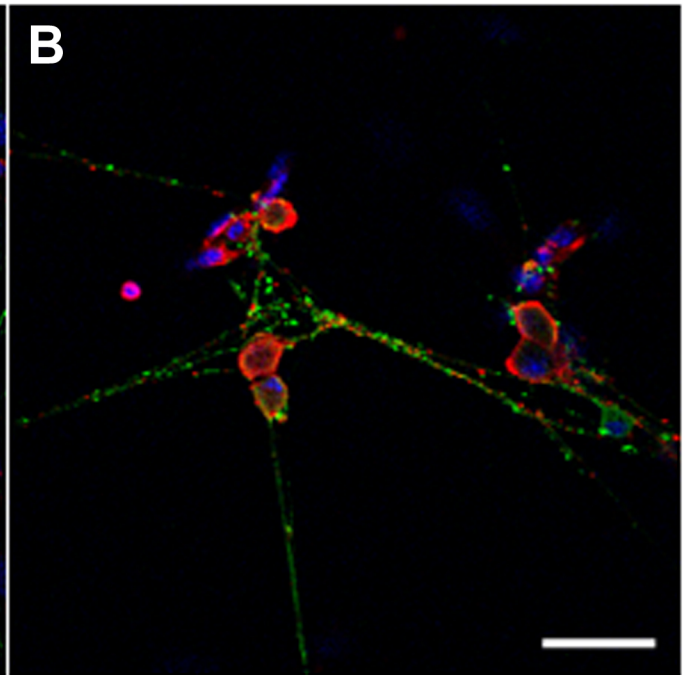
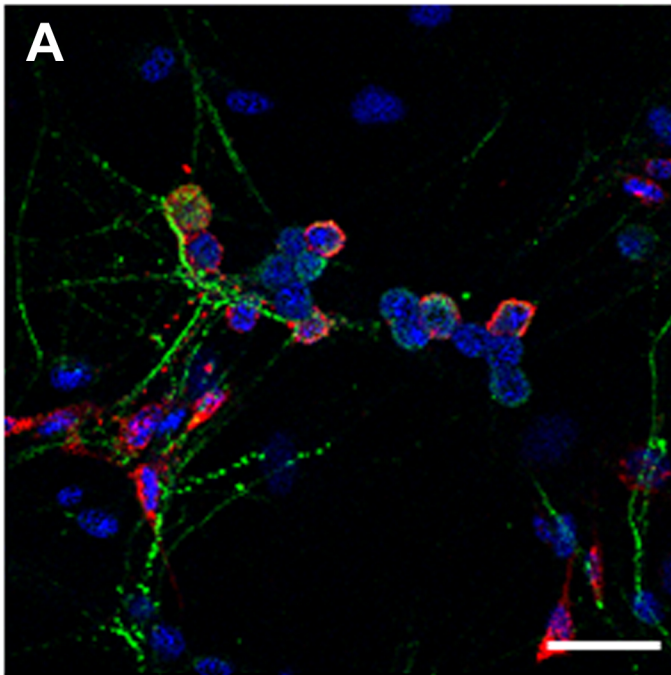


Fig. 14. Confocal images of RV antigen-positive nerve cells with CYD treatment. Confocal images of nerve cells are immunostained for viral antigen (red), actin filaments (green) and nuclei (blue). Nerve cells were infected with 6.3×10^6 FFU/ml of RV. Concentrations of CYD are 1 μ M (B), 2.5 μ M (C) and 5 μ M (D). As for control, vehicle of CYD (A) was used. Bars=100 μ m.

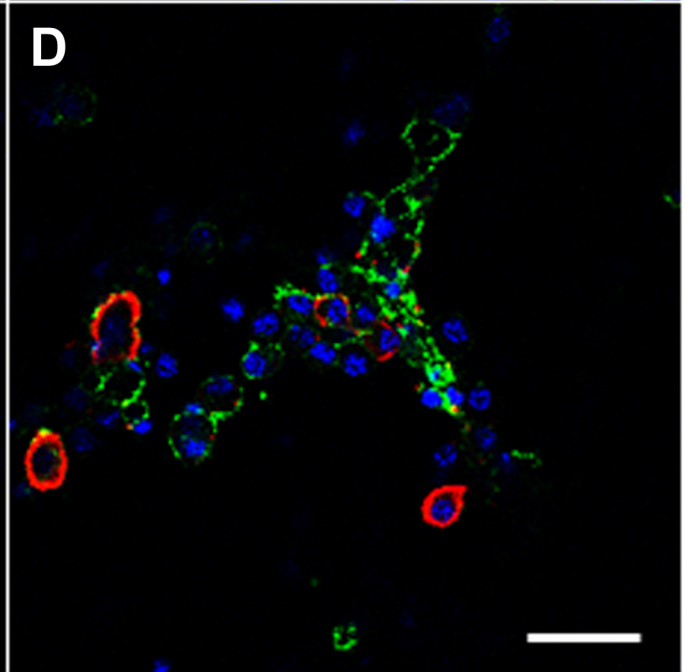
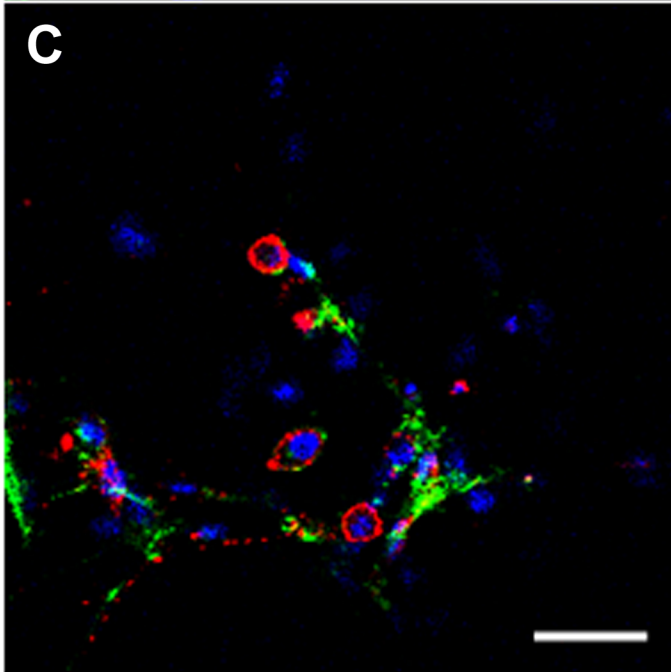
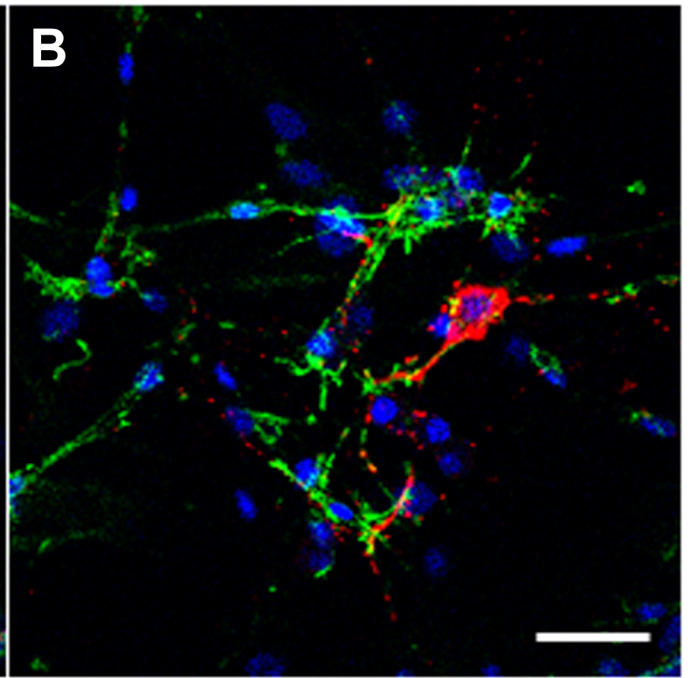
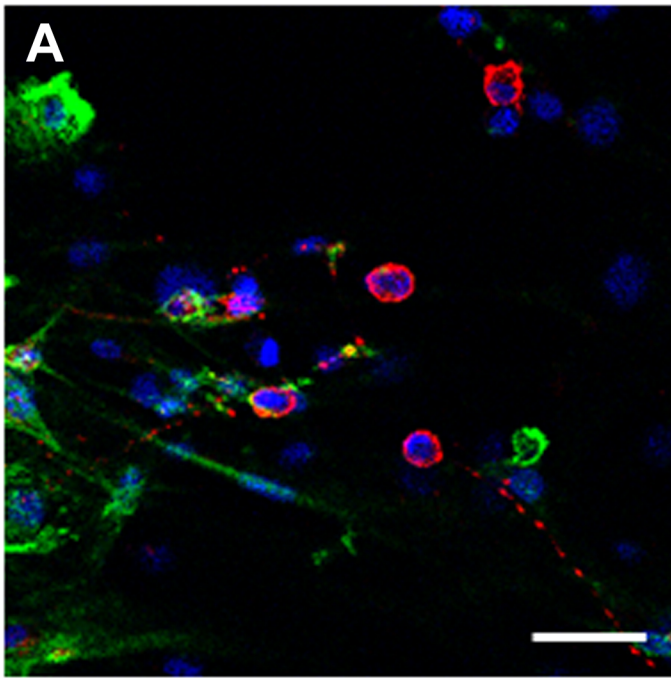
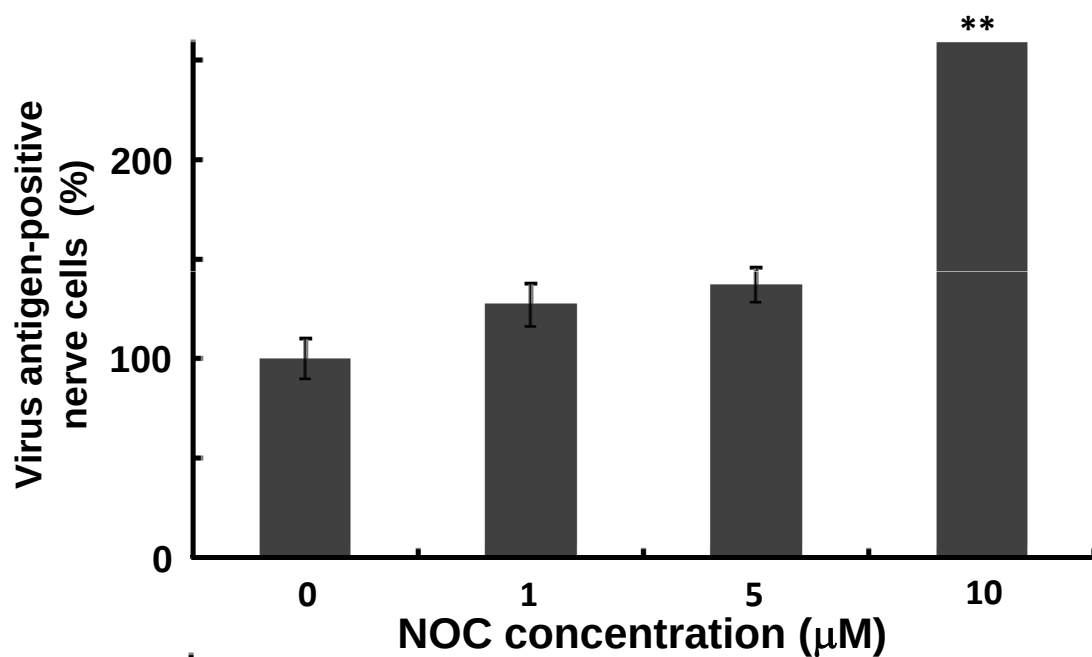
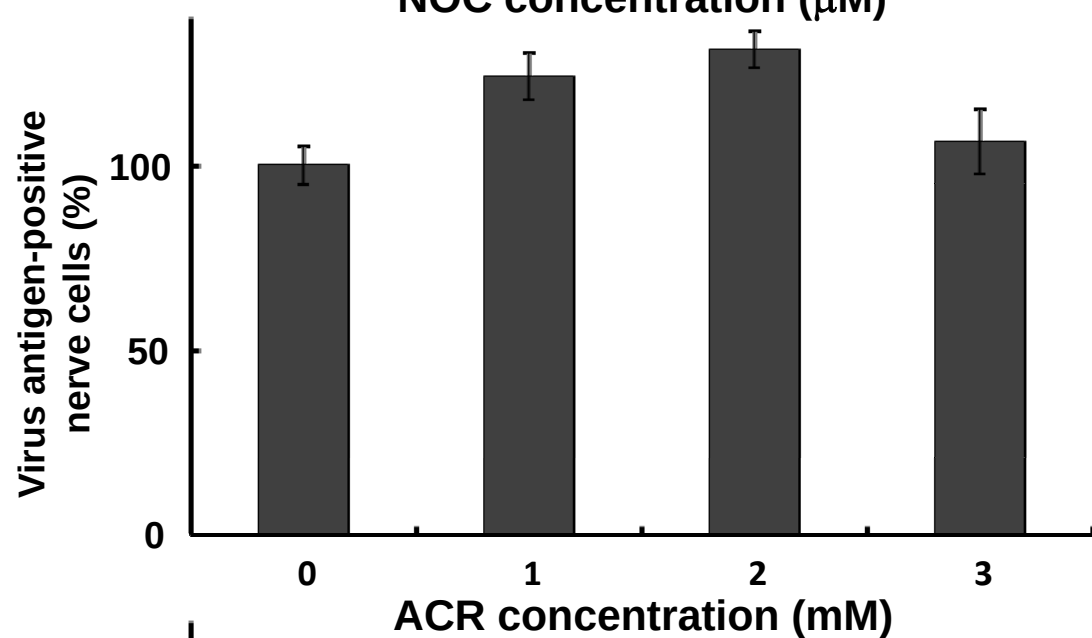
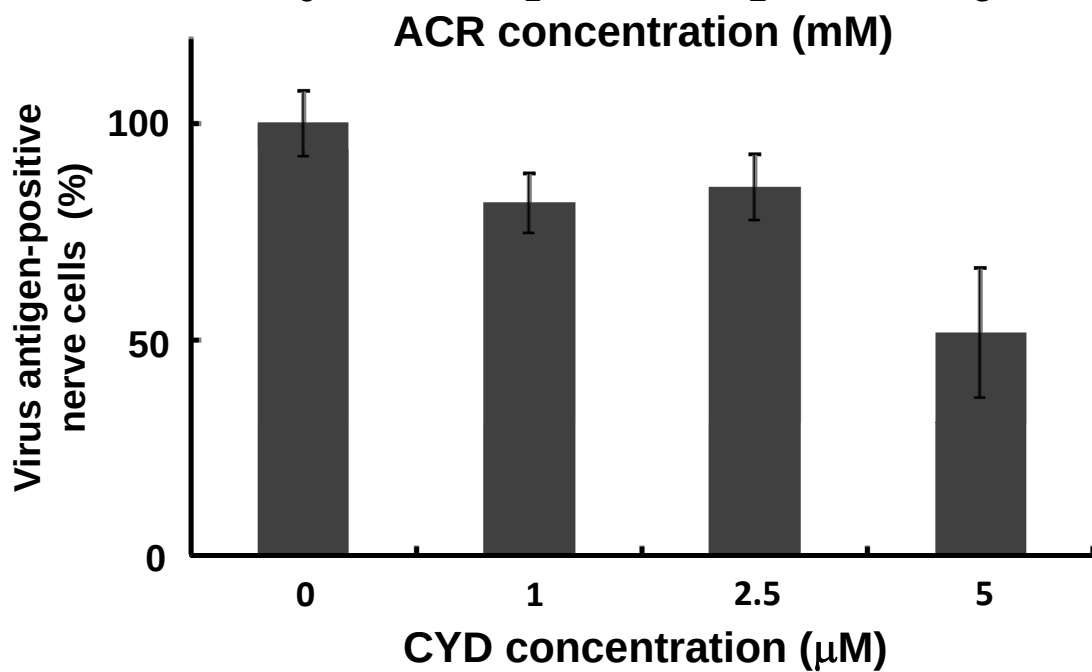


Fig. 15. Effects of cytoskeletal perturbation on RV infection in cultured nerve cells treated with the cytoskeletal inhibitors indicated at the bottom of each panel. Results are expressed as the percentage of viral antigen-positive nerve cells for each concentration of the cytoskeletal inhibitors. Values of the control group are adjusted to 100%. Columns and vertical bars show mean $\pm$ SD. ** $p < 0.01$ vs. controls.

A**B****C**

Discussion

As described in chapter 1, the cultured newborn mouse DRG cells used in this study were composed of nerve cells and non-neuronal cells, and non-neuronal cells were composed of Schwann cells and fibroblasts.

In the present study, the percentages of RV antigen-positive nerve cells were about 15 or 35% at 48 hr p.i., depending on the viral titer in the inoculum, and the maximum percentages were about 40 or 45% at 72 hr p.i. The infectivity of RV in DRG nerve cells was low compared to those of PRV and swine HEV, for which the percentages of antigen-positive nerve cells were 100% for PRV and about 85% for HEV at 48 hr p.i. (chapter 1). The low infectivity of RV was consistent with previous *in vitro* studies (Lycke and Tsiang, 1987; Matsumoto *et al.*, 1974). In the adult rat DRG, not all the nerve cells were infected, in spite of the fact that the culture conditions employed allowed close contact between the virus and cells (Lycke and Tsiang, 1987). An *in vitro* experiment using embryonic neurons also revealed a low infectivity of RV (Matsumoto *et al.*, 1974). A similar experiment using a compartmentalized cell culture system demonstrated that the viral transport of RV in DRG nerve cells was slow (25 mm/day) compared to herpes simplex virus (50 mm/day) (Lycke and Tsiang, 1987; Tsiang *et al.*, 1991). Therefore, the low infectivity of RV in cultured DRG nerve cells in this experiment might have been due not only to low infectivity but also to slow viral transport in cultured nerve cells. These phenomena may be related in part to the long incubation period of the virus in animals and humans.

Non-neuronal cells that coexisted with the nerve cells were also susceptible to RV in this study. The susceptibility of non-neuronal cells has been reported in previous *in vivo* and *in vitro* studies (Castellanos *et al.*, 1997; Murphy, 1977; Rossiter and Jackson, 2007). Intra-axonal transport by fast axonal flow is a well-documented route of RV propagation from the periphery to the CNS and vice versa (Jackson, 2007; Tsiang, 1979). The significance of the infection of non-neuronal cells with RV is not fully elucidated, but the present results and a previous *in vitro* study (Lycke and Tsiang, 1987; Matsumoto *et al.*, 1974) may suggest that neural spread via Schwann cells of peripheral nerves should be considered as another possible route for RV propagation as was evidenced for herpes simplex virus (Shimeld *et al.*, 2001). Non-neuronal cells infected with RV may also support viral entry and replication of RV (Lycke and Tsiang, 1987; Murphy, 1977). Thus non-neuronal cells may play a substantial role in the development of rabies virus infection.

To understand the relationship between RV infection and neuronal cytoskeletal components (microtubules, intermediate filaments, and microfilaments), cultured nerve cells disrupted selectively for these components with specific inhibitors (NOC, ACR and CYD) were exposed to RV. Previous studies indicated that RV requires microtubules and actin for infection and intracellular transport among the nerve cells (Cheung and Terry, 1980; LoPachin, 2004; Lycke and Tsiang, 1987). Those studies used polyclonal antibodies for the detection of the viral antigen and compartmentalized culture system to be infected the RV from axonal ends.

The inoculated virus titer in the present study was higher than those in the previous study (Lycke and Tsiang, 1987) and I used a mouse monoclonal antibody against rabies virus N protein to detect the viral antigens (Minamoto *et al.*, 1994). The signals of N protein produced in the nerve cells were not affected by the disruption of the cytoskeleton. These results may suggest that an intact nerve cell cytoskeleton is required for axonal transport and packaging of proteins and genomes of RV into viral particles, but the whole process of N protein synthesis in the cytoplasm is independent from the cytoskeleton of the host cell.

Summary

RV is highly neurotropic and migrates to the neuronal soma by retrograde axonal transport from nerve terminals, after which it is then taken by anterograde axonal transport to be finally released into the CNS, resulting in lethal encephalitis. DRG are crucial in the initial events of the infection by RV since they can act as gates for the viral entrance into the CNS. In this chapter, I examine cell tropism of RV and the roles of neuronal cytoskeletal components in the production of N protein using cultured nerve cells and non-neuronal cells from DRG of newborn mice. This *in vitro* study demonstrated a low infectivity of RV in nerve cells, susceptibility of non-neuronal cells to RV, and independence of cytoplasmic synthesis of viral N protein from the neuronal cytoskeleton. The present study also suggests that Schwann cells should be considered as another possible candidate supporting RV propagation.

General Conclusion

In chapter 1 of this thesis, I compared neurotropism and the roles of neuronal cytoskeletal components in the neural spread of HEV and PRV using cultured nerve cells and non-neuronal cells from DRG of newborn mice. HEV infected nerve cells but did not infect non-neuronal cells, whereas PRV infected both cell types. By using cytoskeletal inhibitors, it was suggested that infectivity of HEV and PRV within and among nerve cells depended on microtubules and intermediate filaments of nerve cells. Therefore, the viruses may be transported between the cell body and axonal terminals of neurons by fast axonal flow.

In chapter 2, the cell tropism of RV and the roles of neuronal cytoskeletal components in the production of viral protein were examined, using cultured nerve cells and non-neuronal cells from DRG of newborn mice. The infectivity of RV in DRG nerve cells was low, and intracellular RV N protein synthesis was independent from neuronal cytoskeleton. In addition, RV infected non-neuronal cells originated from DRG, suggesting that Schwann cell could be another candidate supporting neural spread of RV.

The present results suggest a diversity of modes in infection and neural spread, and these finding may be important for the development of preventive and therapeutic measures to the diseases caused by the neurotropic viruses.

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Summary in Japanese

神経伝播を示すウイルスは末梢神経系から中枢神経系へと神経を伝播し、致死的な脳炎・脳症を惹起する。神経伝播を示すウイルスとしては、狂犬病ウイルス、単純ヘルペスウイルス、ボルナ病ウイルス、オーエスキー病ウイルス、豚血球凝集性脳脊髄炎ウイルスなどが挙げられる。これらのウイルスの神経細胞内での感染および増殖機構を明らかにすることは、ウイルスの性状を理解する上でも、これらのウイルス感染による疾患の予防・治療法を開発する上でも重要である。

脊髄背根神経節（DRG）は中枢神経系へのウイルスの侵入門戸またはウイルス増殖の場として重要な役割を果たしており、DRG 細胞はこれまでに神経親和性ウイルスの感染および伝播メカニズムを解明するための実験に利用されてきた。当研究室では、これまでに新生マウス DRG 初代培養細胞を用いた *in vitro* の実験系で高病原性トリインフルエンザウイルスの神経伝播の証明を行い、さらに細胞骨格阻害剤を用いてこの伝播が微小管依存性軸索輸送とは異なることを報告している。本研究では神経伝播を示すウイルスとして、豚血球凝集性脳脊髄炎ウイルス（HEV）、オーエスキー病ウイルス（PRV）、狂犬病ウイルス（RV）を選択し、これらの感染および増殖について新生マウス DRG 初代培養細胞を用いた *in vitro* 実験系で検索した。

第 1 章では、HEV の神経細胞における感染および増殖と細胞骨格との関連について、微小管依存性に細胞内輸送をされることが証明されている PRV と比較検討した。まず、新生マウスの DRG 初代培養細胞に HEV または PRV を接種し、その感染性を免疫蛍光染色により検討した。その結果 DRG 神経細胞では、HEV および PRV 共にウイルス抗原が認められた。一方、非神経細胞（シュワ

ン細胞および線維芽細胞)においては、ウイルス抗原が PRV では認められたが、HEV では全く認められなかった。また、微小管または中間径フィラメントの選択的阻害により、DRG 神経細胞における HEV および PRV の感染は有意に抑制された。これらの成績から、PRV と比較して HEV の感染はより強い神経細胞特異性を有すること、および神経細胞での感染は PRV と同様に微小管と中間径フィラメントに依存することが明らかとなった。

第 2 章では、非常に強い神経親和性を有し、末梢神経から中枢神経系へと神経伝播することが報告されている RV を用いて、同様の実験を行った。RV の検出には nucleoprotein (N 蛋白) に対するモノクローナル抗体を用いた。RV 抗原は神経細胞と非神経細胞のいずれにおいても検出されたが、神経細胞の抗原陽性細胞数は HEV や PRV と比較して低値であった。また、微小管、中間径フィラメント、マイクロフィラメントの選択的阻害により、RV 抗原陽性細胞数に有意な変化は認められなかった。以上の成績より、RV 抗原陽性細胞数の低値は、神経細胞内での増殖速度もしくは伝達速度の遅さによるものと推察され、これが動物およびヒトの狂犬病における潜伏期間の長さに関連している可能性が考えられた。RV の感染および輸送が微小管およびアクチンフィラメントに依存することが過去に報告されており、今回の成績と異なる理由については、ウイルス粒子の輸送およびパッケージングにはこれらの細胞骨格成分が関与するが、ウイルス N 蛋白は微小管およびアクチンフィラメントとは独立した機構により合成される可能性が推察された。また、RV の非神経細胞への感染が認められたことから、これらの細胞が RV の神経細胞内での感染および増殖の支持細胞として関与している可能性と、シュワン細胞における感染が RV の神経伝播に関与する可能性が考えられた。

以上の結果は神経親和性ウイルスの感染および伝播様式の多様性を示唆して

おり、これらのウイルス感染症の予防・治療法を開発する上で重要な知見と考えられた。