Application of recombinant CDVs as leishmania vaccine and delivery vector

（組換えイヌジステンパーウイルスのリーシュマニアワクチンおよびデリバリーベクターとしての応用）

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Contents

GENERAL INTRODUCTION

Canine distemper virus (CDV) is a non segmented single stranded RNA virus, which belongs to *morbillivirus*. Morbillivirus is a family of *Paramyxoviridae*, in which there are many serious pathogenic viruses to human, companion, domestic, laboratory and wild animals. The family *Paramyxoviridae* is further divided into two subfamilies: subfamily *Paramixovirinae* and subfamily *Pneumovirinae*. Subfamily *Paramyxovirinae* contains five genuses: genus *Respirovirus*, *Morbillivirus*, *Rubulavirus*, *Avulavirus* and *Henipavirus*. Genus *Respirovirus* contains Human parainfluenza virus type 1 and type 3 (PIV-1 and PIV-3), Sendai virus (SeV) and Bovine parainfluenza virus type 3 (BPIV-3). These viruses cause respiratory tract illnesses in the natural host. Genus *Morbillivirus* contains Measles virus (MV), Rinderpest virus (RPV), Canine distemper virus (CDV), peste des petits ruminant's virus (PPRV), Phocine distemper virus (PDV), Dolphin morbillivirus (DMV) and Porpoise's morbillivirus (PMV). These viruses cause systemic symptoms such as bronchitis, pneumonia, diarrhea and encephalitis. The genus *Rubulavirus* contains human parainfluenza virus type 2, 4a and 4b, Mumps virus, and Simian virus 5. Genus *Avulavirus* contains avian paramyxoviruses and Newcastle disease virus. Genus *Henipavirus* is classified recently and known to contain emerging viruses, Hendra virus and Nipah virus.

Canine distemper (CD) caused by CDV is one of the lethal infectious diseases in dogs and many other carnivores, including raccoons, ferrets, and foxes. CDV induces fever, gastroenteritis, pneumonia, conjunctivitis and encephalomyelitis and showed high mortality in pappies. Transmission generally occurs via an aerosol-droplet route, direct contact, or possibly by contact with contaminated objects. The virus is shed in the

feces and urine of infected individuals and some evidence exists for trans placental transmission. The usual route of infection is through the upper respiratory tract, following inhalation of the virus. Dogs in recovery may continue to shed the virus for several weeks after symptoms disappear, but they no longer shed the virus once they are fully recovered. Macrophages (cells that ingest foreign disease-carrying organisms, like viruses and bacteria) carry the inhaled virus to nearby lymph nodes where it begins replicating (reproducing). It spreads rapidly through the lymphatic tissue and infects all the lymphoid organs within 2 to 5 days. By day six to nine, the virus spreads to the blood (viremia). It then spreads to the surface epithelium of the respiratory, gastrointestinal, urogenital and central nervous systems, where virus begins damaging that causes the symptoms. Recently, von Messling and colleagues showed more precise pathway of CDV infection using ferrets and recombinant CDV expressing enhanced green fluorescence protein [von Messling, 2004]. After experimental inoculation of ferrets intranasally, first it infected rapidly and massively circulating B and T cells. Second, it took over and damaged secondary lymphatic organs including spleen, lymph nodes, and gut-associated and mucosal lymphoid tissues. Third, it infected most thymocytes. In contrast, replication in epithelial cells was initially not detectable, but substantial before host death. Thus, CDV initially infects lymphocytes and massively replicates therein, thereby causing immunosuppression, and preparing systemic invasion and host escape.

Live attenuated vaccines have been used successfully for many years to control CD in the world. However, there were CD-outbreaks in several parts of the world [Blixenkrone-Møller, 1992; Bolt, 1997; Haas, 1997; Ek-Kommonen, 1997; Jozwik, 2002]. Similarly, incidence of CD both in unvaccinated and vaccinated dogs had rapidly

increased in 1989 in Tokyo area [Gemma, 1996b]. Clinically, the affected dogs showed neurological, respiratory and gastrointestinal signs of varying degrees. Some dogs showed severe central nervous system signs and mild respiratory signs but no gastrointestinal signs. Comparison of the antigenicities between the Onderstepoort vaccine strain and Japanese field isolates of CDV suggested that the generation of different types of CDV population have been associated with the current outbreaks of CD in Tokyo. Okita and colleagues also suggested that there were two distinct types of CD, with or without cytopahogenic effects in the gastrointestinal tissues, in Japan [Okita, 1997]. Following these clinical and serological studies, molecular approaches identified the differences of the vaccine strains and Japanese recent field isolates of CDV [Gemma, 1996b; Iwatsuki, 1997, 2000]. Numbers of CDV strains were successfully isolated from affected dogs in recent years. On their genetic and antigenic characteristics, recent field isolates were clearly distinguishable from the vaccine strains of CDV, and Japanese isolates formed one group which is phylogeneticaly distinguishable from European or American groups [Iwatsuki, 1997, 1998, 2000; Yoshida, 1998].

CDV also appears to be an increasing problem in wildlife. It is known that CD with varying morbidity and mortality in distributed in the world. The epizootic of European pinniped in 1988, it came to international attention that thousands of Baikal seals (Phoca sibirica) had died in Lake Baikal from late 1987 to late 1988 [Likhoshway 1989; Osterhaus, 1989a, b, c]. Subsequent identification of a morbillivirus isolated from Baikal seals revealed that the causative agent was a strain of CDV [Mamaev, 1995, 1996; Ohashi, 2001]. Recently, CDV had been implicated in the deaths of Caspian seals (Phoca caspica), and from one of the dead seals, a part of CDV genome was detected [Ohashi,

2001]. This virus was different from the virus which caused disease in Lake Baikal seal in 1987 and from other viruses in circulation in different parts of the world [Forsyth, 1998]. In 1992, several species of large cats including lions (Panthera Leo), tigers (Panthera Tigris) and leopards (Panthera pardus) died of CDV infections in American zoos [Appel, 1994]. This outbreak was unusual as CDV was not previously thought to cause a clinical disease in *Felidae*. The outbreaks of distemper-like disease among captive and feral large felids were most likely initiated by interspecies transmission of CDV from local feral (non-felid) carnivores [Harder, 1996]. In 1994, lions in Serengeti National Park, in Tanzania, died in large numbers, and CDV was identified as the causative agent [Morel, 1996; Roelke-Parker, 1996]. Additionally, 55% of lions of the Masai Mara which forms continuous ecosystem with the Serengeti Plain had anti-CDV antibodies between October 1994 and February 1995 [Kock, 1998]. During the epizootic in lions in Serengeti, a group of spotted hyenas (Crocuta crocuta) also died of CDV infection again, although the hyena was not thought to be a natural host for this virus [Haas, 1996]. Moreover, CDV infections were reported in mustelids in Germany [van Moll, 1995], in brown bears (Ursus arctos arctos) in Italy [Marsilio, 1997], in African wild dogs (Lycaon pictus) in Tanzania [Creel, 1997], in Coyotes (Canis latrans) and black bears (Ursus americanus floridanus) in USA [Cypher, 1998; Dunbar, 1998], and in Binturongs (Arctictis binturong) in Korea [Hur, 1999].

The pathology of distemper has been extensively described by many pathologists over the past century [Innes, 1952; Saunders, 1973]. Although the spectrum of the lesion appears to be wide, the neuropathology of spontaneous distemper is remarkably constant. The variability of the neuropathology is largely due

to the evolution of the lesions when the disease progresses. The so-called "old encephalitis" [Lincoln. 1971, 1973] in naturally affected dogs, a disease which differs from the chronic form of common nervous distemper [Vandevelde, 1980] and closely mimics measles virus induced subacute sclerosing panencephalitis (SSPE) in people, are observed although the cases are exceedingly rare. As similar to spontaneous infections, the CDV R252 [McCullough, 1974] and A75/15 [Summers, 1979] strains cause multifocal lesions in the grey as well as white matter of the central nervous system (CNS) [Innes, 1952; Saunders, 1973; Frauchiger, 1968; Bestetti, 1978]. In the grey matter, CDV infects neurons which can lead to neuronal necrosis and even encephalomalacia [Lisiak, 1979; Krakowka, 1978]. Neuronal infection can also be very widespread with remarkably little evidence of cytolysis. It has been known for more than a century that the white matter lesions in distemper are characterized by selective loss of myelin sheaths [Bestetti, 1978]. The demyelination lesions is responsible for sever neurological signs and that caused by CDV are thought to be a model for human demyelinating conditions such as multiple sclerosis [Appel, 1981; Haile, 1982]. However, the precise mechanism of CDV spread in CNS is still unknown. In chapter 1, the author analyzed the tropism of the recent isolated CDV in hippocampus as a model for spreading CDV in CNS of affected dogs using a recombinant CDV expressing enhanced green fluorescent protein.

Paramyxoviruses contain non-segmented single stranded RNA genomes of negative polarity, and they replicate entirely in the cytoplasm. The genomes of morbilliviruses are approximately 16 kb in length, and the genomes contain six tandemly linked genes. A lipid envelope containing two surface glycoproteins, fusion (F)

protein and hemagglutinin (H) protein, surrounds the virions. F protein is responsible for virus-cell fusion and cell-cell fusion which facilitates virus penetration into cells and viral spread between cells. H protein interacts with cellular receptors and play a role of virus attachment to the cells. CDV H protein can cause agglutination of dog erythrocytes but lacks detectable neuraminidase or esterase activity. These two envelope proteins are of primary importance in inducing neutralizing antibodies and immunity against reinfection. The viral matrix (M) protein localizes at the inner layer of plasma membrane of the infected cells and is involved in virus budding from the infected cells. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleo- (N), phospho- (P) and large (L) proteins, which initiate intracellular virus replication. The RNA genome are tightly associated with N protein to form ribonucleocapsid (RNP) complex with P and L proteins, and the RNP but not naked RNA can be the template for transcription and replication. The viral P and L proteins form RNA-dependent RNA polymerase complex, and are responsible for viral transcription and replication. The RNP is packaged in viral particle, highly attached viral glycoproteins and then released from the cells. Compared to the RNP in the intracellular environment, its morphologic structure in viral particle has not been investigated extensively. Although the RNP penetrates cytoplasm after the infection, it is not clear how adequate morphologic structure of RNP is packaged in viral particle in order to start transcription and replication. In chapter 5, author investigated morphologic structure of CDV genome packaged in viral particle.

All paramyxovirus P proteins are likely to be oligomeric, containing heptads sequence repeats in the central region (approximately 340-410aa) of the molecule,

characteristic of helical coiled coils [Curran, 1995]. The structure of this region of the SeV P protein has determined and is a tetrameric coiled [Tarbouriech, 2000]. The extreme of C terminus of P protein is critical for nucleocapsid binding, as SeV with the shorted C terminus eliminates or dramatically decreases the formation of N-RNA. The final 50 amino acids are predicted to form a trihelical structural motif in many paramyxoviral P proteins, including MV [Curran, 1995]. Recent crystal structure analysis of this domain of MV P protein [Johansson, 2003] showed it to be a compact bundle of three alpha-helices. For MV, the C-terminal region of P protein, including the putative coiled coil and down stream sequences, can bind to the N protein [Huber, 1991; Liston, 1995; De, 2000; Longhi, 2003]. P protein has been shown to have multiple functions. The P protein is activated by phosphorylation and binds to N, L and viral RNA to form the RNP complex [Robbins, 1979]. Serine/threonine phosphorylation of P protein is carried out by cellular kinases, primarily casein kinase II [Das, 1995]. Although the RNA-dependent RNA polymerase L protein is believed to contain all catalytic activities [Poch, 1990], P protein is essential for viral RNA synthesis. Its interaction with the N protein keeps RNP complex in a soluble form and imparts specificity to the N protein to encapsidate viral, but not cellular, RNAs. Moreover, in SeV, it was reported that binding of P to L allowed efficient interaction of L with the N-RNA template [Horikami, 1992], and that P protein binds N-RNA template independent of its complex formation with L protein and activates transcription [Curran, 1996]. For VSV P protein, replacement of single amino acids in the C-terminal region resulted in P mutant that were defective in transcription but supported replication, suggesting that the transcriptase and replicase complexes may not be identical [Das, 1997, 1999]. On the other hand, Yoneda and colleagues reported that RPV H protein is important for host

specificity and P protein has a key role in the replication of the virus in the cross species infection and pathogenicity using an animal model of rabbit and recombinant viruses based on the RPV-L strain [Yoneda, 2004]. MV V protein, which is translated from P gene, was reported to show the inhibition of IFN-γ reaction in MV-infected cells [Takeuchi, 2003]. In Chapter 4, to understand the multifunctional P protein, author established and characterized monoclonal antibodies (MAbs) against CDV P proteins. One of the MAbs allowed author to analyze the morphologic structure of CDV genome in Chapter 5.

It had been impossible to recover genetically engineered recombinant viruses for Mononegaviruses because unlike positive sense RNA viruses, the naked RNA of Mononegaviruses alone can not be the templates for viral transcription and replication. In 1994, Conzelmann and colleagues [Schnell, 1994] generated recombinant rabies virus (RV), demonstrating for the first time the feasibility of producing a negative-sense RNA virus entirely from cloned cDNA. Cells were cotransfected with protein expression constructs for the L, P and N proteins and with a cDNA construct encoding the full-length RV antigenome, all under control of the T7 RNA polymerase promoter. Infection with recombinant vaccinia virus, which provided T7 RNA polymerase, was the final step needed to produce infectious RV. The key element to this success was the synthesis of a positive-sense antigenomic RNA from cloned DNA. Positive-sense antigenomic RNA, in contrast to negative-sense genomic RNA, cannot hybridize to positive-sense mRNAs encoding the L, P and N proteins and thus does not interfere with virus generation. Moreover, the genomic RNAs of some negative-sense RNA viruses contain stretches of uridine residues followed by hairpin structures that resemble T7 RNA terminator elements, which may cause premature abortion of T7 RNA polymerase

transcription [Whelan, 1995]. Since the initial report by Schnell and colleagues (1994), the generation of an ever-growing number of Rhabdoviruses and Paramyxoviruses by reverse genetics was carried out [MV: Radecke, 1995; Schneider, 1997; Takeda 2000; RPV: Baron, 1997; CDV: Gassen 2000; Fujita, 2002; Parks, 2002; Plattet, 2004; SeV: Garcin, 1995; Kato, 1996; hPIV3: Durbin, 1997; Hoffman, 1997; bPIV3: Haller, 2000; SV5: He, 1997; Mumps V.: Clarke, 2000; hPIV2: Kawano, 2001; NDV: Peeters, 1999; Romer-Oberdorfer, 1999; Krishnamurthy, 2000; hRSV: Collins, 1995; bRSV: Buchholz, 1999]. Refinements of the original rescue procedure included the expression of T7 RNA polymerase from stably transfected cell lines [Radecke, 1995], protein expression plasmids [Lawson, 1995] or heat shock procedures to increase rescue efficiencies [Parks, 1999] were performed. Recently, Ebola virus, a member of the family *Filoviridae*, was also generated from cDNA [Volchkov, 2001; Neumann, 2002]. Recently, we succeeded the establishment of reverse genetics of the CDV Yanaka strain [Fujita, 2002], which was isolated in Japan from a dog clinically diagnosed with distemper, and produced the recombinant CDVs which express enhanced green fluorescence protein (EGFP) and firefly luciferase [Fujita, 2002]. Author utilized the EGFP-CDV in chapter 1. And using this reverse genetics, author constructed recombinant CDVs in chapter 2 and 3.

Negative-sense RNA viruses have several biological features that make them promising candidates as vaccine or targeting vectors. Most importantly, they do not replicate through DNA intermediates, so that integration of their genomes into the host cell genome is a remote possibility. Also, most members of this virus group grow to high titers, accommodate additional genetic materials and express the foreign peptides or

proteins at high levels. Moreover, strong humoral and cellular immune responses have been observed after immunization with negative-sense RNA virus vectors. Morbillivirus are also used as recombinant vaccines expressing foreign genes. RPV causes severe disease in cattle, leading to appreciable economic losses. An effective live attenuated vaccine is available that provides lifelong protection, but only one RPV serotype exists, so that vaccinated animals cannot be distinguished from those that developed immunity due to a natural infection. To overcome this problem, Walsh and colleagues generated recombinant RPV that expressed genetic markers, such as EGFP or the influenza virus HA [Walsh, 2000]. Cattle immunized with the recombinant viruses expressed anti-GFP or anti-HA antibodies and developed protective immunity against RPV [Walsh, 2000]. PPRV causes an infection in sheep and goats that clinically resembles RPV infections. Both viruses are members of the *Morbillivirus* genus and their genetic relationship allowed the generation of a recombinant RPV that expressed the PPRV F and H proteins [Das, 2000]. In contrast, no virus was recovered when only one glycoprotein was replaced. Further studies demonstrated that the RPV/PPRV chimeric virus was attenuated in cell culture and protected goats against infection with wild-type PPRV [Das, 2000]. MV-based HIV vaccine was constructed and showed the potential to generate a neutralizing antibody in macaque monkey [Lorin, 2004].

Leishmaniasis is distributed in parts of 88 countries with 12 million people in tropical and subtropical regions. Number of new cases of leishmaniasis each year in the world reaches more than one million. The World Health Organization documented in 1993 that Leishmaniasis was one of the six major tropical diseases of developing countries. Leishmaniasis is caused by infection with parasite protozoa *Leishmania* and

has a large spectrum of zoonotic diseases in human and animals, ranging from self-curing skin ulcers to the severe and lethal pathologies of visceral form. Leishmaniasis is a complex disease with various symptoms, including cutaneous, mucocutaneous and visceral leishmaniasis. For the patients at the late stage, there are no effective treatment and drugs. The parasites are naturally transmitted by blood-sucking sand flies among reservoir animals including rodents and dogs. Humans are accidental hosts transmitted from the animals. In the previous reports, inducing cell-mediated immunity is more important to cure and protect from leishmaniasis than serological one [Vanloubbeeck, 2004]. Epidemiologically, the elimination of canine leishmaniasis has been correlated with a decreased prevalence of disease in human, reported in Brazil [Dietze, 1997]. There are, however, no effective vaccines for human and even dogs [Vanloubbeeck, 2004]. The leishmania strains that dogs are host or reservoir, and have infectivity between human and dogs are, *Leismania donovani chagasi*, prevailed in older children or young adults in eastern India, Bangladesh, and Pakistan, *Leishmania infantum*, prevailed in children between ages 1 and 4 in Mediterranean regions, sub-Saharan and East Africa, the Middle East, and China, *Leishmania major* ZCL (zoonotic cutaneous leishmaniasis) spreaded in large urban areas in the Middle East, the Mediterranean region and the Indian subcontinent, *Leishmania tropica* in eastern Mediterranean regions, including Iran, Iraq, India and Israel, *Leishmania braziliensis* in Brazil, Peru, Ecuador, Bolivia, Venezuela, Paraguay, and Columbia and *Leishmania peruviana* in Peruvian Andes only at attitudes of 900 to 3000 meters. In this list, *L. infantum* is the leishmania strain which is known as one of the most important strains between human and dogs. *L. infantum* causes visceral leishmaniasis and the lesions spread in systematically including epicutaneous and

visceral organ. In chapter 3, author constructed and evaluated the recombinant CDV for leishmania vaccine.

The brain progressively accumulates oxidative. Free radicals attack all cell constituents: lipids, proteins and nucleic acids, often with adverse consequences. Most of creatures have antioxidant enzymes to protect themselves against free radical damages or reduce the adverse products which reacted with free radicals. Oxidative stress causes some neurological diseases directly or indirectly. Perkinson's disease is a common movement disorder that is due to the destruction of neurons in the substantia nigra pars compacta of the basal ganglia. The principle cause of Altzheimer's disease is not known but free radical reacted substances cause of it. Superoxide dismutases (SODs) are the first and most important line of antioxidant enzyme defense systems against reactive oxygen species and particularly superoxide anion radicals. At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described [Zelko, 2002]. Two isoforms of SOD have Cu and Zn in their catalytic center and are localized either to intracellar cytoplasmic compartments (CuZn-SOD or SOD-1) or to extracellar elements (EC-SOD or SOD-3) [Skrzycki, 2004]. SOD1 has a molecular mass of about 32 kDa (human) and has been found in cytoplasm, nuclear compartments and lysosomes of mammalian cells [Chang, 1988; Keller, 1991; Crapo, 1992; Liou, 1993]. Antioxidant gene therapy had been already major methods to protect against neurological disorders experimentally [Davis, 1997; Asanuma, 1998; Barkats, 2002]. But the delivery system of antioxidant gene had been still developing by many roots or vectors because of the tight brain protection, blood brain barrier (BBB). Viral vectors are expected to be one of the good tools to

overcome BBB and reach the target cells in CNS by their kinetics. In chapter 2, author constructed the recombinant CDV as a delivery vector.

CHAPTER 1

Visualization of Canine Distemper Virus Infected in Dog Hippocampus using Enhanced Green Fluorescent Protein

SUMMARY

Canine distemper virus (CDV) infection of dogs causes an acute systemic disease and often induces central nervous system (CNS) symptoms. In this study, the author analyzed the mechanism of CDV propagation and spread in dog hippocampal slices by using recombinant CDV expressing enhanced green fluorescence protein (EGFP-CDV). The CDV infection and propagation mainly occurred in cornus ammonis (CA) regions. Neurons are more effectively infected with the EGFP-CDV than astrocytes and oligodendrocytes. The EGFP-CDV was observed to transport from a neuron to the other through synapses, and a part of synapses of infected neuron mediated the transmission. The viral envelop proteins F and H were distributed in the whole neurons but M protein is mainly located at soma and partially at dendrites and synapses. Electron microscopic analysis demonstrated that ribonucleocapsid protein (RNP) is sorted to synaptic terminals and viral particles were visualized at extrasynaptic regions. These data suggest that CDV may be transmitted to synapses of neurons via extrasynaptic regions.

INTRODUCTION

Canine distemper (CD) is a contagious, often fatal, multisystemic viral disease that affects the respiratory, gastrointestinal and central nervous systems. Distemper is caused by the canine distemper virus (CDV) which is a nonsegmented and single strand RNA virus, and belongs to the genus *Morbillivirus*, the family *Paramyxoviridae*. CDV affects domestic dogs and many other carnivores, including raccoons, ferrets, and foxes. The development of a vaccine in the 1950s led to a dramatic reduction in the number of infected domestic dogs. However, recently outbreaks of CD were occurred in Japan, and the affected dogs were clinically classified into two groups; dogs with respiratory and gastrointestinal signs associated with central nervous system (CNS) signs, and those with CNS signs alone [Gemma, 1996b; Okita, 1997]. It was noted that most of the affected dogs which showed only CNS signs had vaccine history [Gemma, 1996b]. These results suggested the presence of different types of CDV population which was associated with the current outbreaks of CD. In general, CDV infection in CNS is considered to be transmitted by cell-to-cell infection, and causes demyelination after oligodendrocyte infection [Schobesberger, 2002]. CDV propagates as much severer in white matter as in gray matter of dog brain [Vandevelde, 1995] and the infection in hippocampus was observed [Braund, 1981]. However, there had been a few reports on the mechanism of CDV spread in the CNS.

Subacute sclerosing panencephalitis (SSPE) is known as a fatal disease and is considered to be caused by MV after long period infection in CNS. In SSPE, CNS infection develops in the presence of high titers of antiviral antibodies, and the isolated viruses are defective in budding [Dubois-Dalcq, 1976]. Mutations accumulate in the virus genome, especially in the F and M genes, and transcription of envelope genes is reduced

by an altered transcription gradient [Baczko, 1986, Cattaneo, 1988, Taylor, 1991, ter Meulen 1997]. Studies of MV neurotropism have been mainly used to generate a mice model for CNS infection. Following the identification of CD46 as a receptor for the Edmonston B strain of MV, transgenic mice which express the receptor have been developed [Rall, 1997; Evlashev, 2000]. The other approach has been done using rodent brain-adapted strains of MV by repeated passages in the CNS [Castro, 1972]. These approaches of MV, however, are based on experimental animal models which are not natural hosts and it is not clear whether natural transmission in the CNS is reflected in the models.

Recently, Fujita and colleagues succeeded the establishment of reverse genetics of the CDV Yanaka strain [Fujita, 2002], which was isolated in Japan from a dog clinically diagnosed as distemper, and produced the recombinant CDVs which express enhanced green fluorescence protein (EGFP) and firefly luciferase [Fujita, 2002]. In the chapter 1, author investigated how CDV is propagated and spread in the CNS of a dog brain by using the recombinant CDV expressing EGFP and dog hippocampal slices in keeping the higher order structure, and whether recombinant CDV can be utilized for a delivery viral vector.

MATERIALS AND METHODS

Virus

Enhanced green fluorescent protein gene integrated canine distemper virus (EGFP-CDV) was based on the Yanaka strain, which was isolated in Japan from a dog clinically diagnosed as distemper [Gemma 1996b], and successfully rescued by using the reverse genetics technique [Fujita, doctor thesis 2002]. EGFP-CDV was established by Fujita and colleagues [Fujita, doctor thesis 2002]. The EGFP-CDV was propagated in B95a cells, and viral titration was carried out in duplicate in 96-well plates by limiting dilution method [Iwatsuki, 2000].

Preparation of dog hippocampal slice cultures

Hippocampal slice culture was performed according to the method described by Stoppini and colleagues [Stoppini, 1991]. A 6-month-old healthy beagle dog unvaccinated with CDV was anesthetized and the hippocampus was aseptically removed (Fig. 1A) and placed into ice-cold cutting solution (120mM CholineCl, 3mM KCl, 8mM $MgCl_2$, 1.25mM NaH_2PO_4, 20mM Glucose, 26mM $NaHCO_3$). Hippocampus was excised and sectioned transversely to the septotemporal axis at about 250μm by a vibratome (Leica) in ice-cold cutting solution. Slices were placed into ice-cold artificial cerebrospinal fluid (ACSF: 124mM NaCl, 2.5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 1.25mM NaH_2PO_4, 20mM glucose, 26mM $NaHCO_3$) with the aid of 95% O_2 and 5% CO_2 bubbling for about 1 hr. Hippocampal slices were cultured with modification from Stoppini's method [Stoppini, 1991]. Slices were then placed on a transparent porous membrane (pore size 0.4μm, Millicell-CM filter inserts, Millipore), and cultured at the interface between medium, and 5% CO_2-enriched atmosphere at 35°C. Several slices of the

hippocampus were placed on a culturing membrane and which were set in a 6-well plate. The culture medium was composed of 25% heat inactivated horse serum (Gibco, BRL), 75% DMEM-Hanks (Sigma-Aldrich), supplemented with 0.65% sucrose and 100 units of penicillin/streptomycin.

Virus inoculation and EGFP expression analysis

The hippocampal slices were incubated for 3 weeks before infection for stabilization. Forty μl virus solution of EGFP-CDV at the concentration of $1x10^5$ $TCID_{50}$/ml was dropped on the surface of each slice and readily removed (Fig. 1B). The infections were performed at room temperature. The fluorescence demonstrated by EGFP expression was observed with an inverted confocal laser microscope (Fluoview FV500, Olympus).

Immunohistochemistry

The hippocampal slices were fixed in 0.1M phosphate buffer containing 0.1% glutaraldehyde and 4% formaldehyde in at pH7.4 for 3 hrs at 4°C. Then the slices were permiabilized at room temperature for 12 hr in PBS containing 0.4% Triton X-100, and blocked by 1% (vol/vol) heat inactivated horse serum in PBS for 1 hr. Brain cell specific monoclonal antibodies (MAbs); microtubule-associated protein 2ab (Map) for neuron, glial fibrillary acidic protein (GFAP) for astrocyte and C-type natriuretic peptide (CNP) for oligodendrocyte, were purchased by Sigma-Aldrich. The whole slices were incubated for 8 hr at 4℃ with each MAb which was diluted at 1:200 with PBS. Three times consecutive washing in PBS was performed at room temperature for 30 min each. After 6 times washing for 30 min each in PBS, slices were reacted with rhodamine-conjugated

anti-mice IgG (ICN Biomed) at 1:300 with PBS for 6 hrs at 4℃. The slices were mounted at slides and visualized with confocal microscopy.

Electron microscopic analysis of EGFP-CDV infected hippocampal cells

Hippocampal slices were fixed in 0.1 M phosphate buffer (pH7.3) including 2% formaldehyde, 2.5% glutaraldehyde for 1hr. The slices were post-fixed in 1% osmium in the phosphate-buffer for 1 hr, dehydrated and embedded in Epon (TAAB). Fifty nm of ultra thin sections were prepared by ultra microtome (Leica), stained with 2% Uranyl acetate in 70% Ethyl alcohol and lead citrate. The sections were examined with 1200EX electron microscope (Jeol).

RESULTS

Preparation of hippocampal slices infected with EGFP-CDV

Hippocampus of a 6-month-old healthy dog unvaccinated with CDV, was collected and sliced by vibratome under ice-cold cutting solution (Fig. 1). Sliced hippocampi were immediately placed on specific tissue culture system and maintained in keeping the higher order structure in 5% CO_2 condition. The slices were not used for experiments for 3 weeks until surface cells were replaced because the cells at slice cutting surface were damaged and died just after cutting [Stoppini, 1991]. Sequentially, the cell population and hippocampal conformation were confirmed to maintain under the light microscope. The EGFP-CDV was inoculated on the whole surfaces of hippocampal slices and readily aspirated (Fig. 1B). Although DNA or proteins into the slices have been generally introduced by local injection directly into the sliced tissues, the surface inoculation was undertaken because the slice was derived from a natural host animal of CDV.

Propagation and spread of EGFP-CDV in dog hippocampal slices

The propagation and spread of the EGFP-CDV was evaluated under the confocal laser microscope. After the inoculation, EGFP-fluorescence in several cells was detected as early as 24 hr. At 30th day, EGFP-positive cells were mostly detected in cornus ammonis (CA) regions (CA1, CA2, and CA3), hippocampal base and dentate gyrus (Fig. 2A and B). The positive cells in 6 of 10 slices were detected in CA1, in 7 of 10 slices in CA2, in 4 of 10 slices in CA3, in 5 of 10 slices in hippocampal base and in 3 of 10 slices in dentate gyrus (Fig. 2C). The localization of EGFP-positive cells was shown to be consistent with pyramidal cell layer, in which neuron are more accumulated than

in other regions.

Identification of cell type of EGFP-CDV infected cells in the hippocampal slices

To determine the cell type of EGFP-positive cells in the hippocampal slices, the author performed immunohistochemistry using specific MAbs to maker proteins of neuron, oligodendrocyte and astrocyte (Fig. 3). EGFP-positive cells (75-173 cells) were randomly picked up and classified to neuron, oligodendrocyte and astrocyte. A total of 82% EGFP-positive cells were identified as neuron, whereas oligodendrocyte and astrocyte were 4% and 17% respectively. Since the cell population of neuron in the hippocampus as well as other brain parts was extremely lower than those of oligodendrocyte and astrocyte [Zurbriggen, 1984], EGFP-CDV is apparently selected to infect neuron.

Transmission of EGFP-CDV in the hippocampal slices

Within 24 hrs post infection, EGFP fluorescence was observed in single neural cells or in several neural colonies formed (Fig. 4A). When B95a cells, which are usually used for in vitro experiments of CDV, are infected with EGFP-CDV at low titer, it takes at least 24 hrs to produce the EGFP-fluorescence (data not shown). This result indicates that neuron also has a high capacity of CDV replication as well as the cell lines derived from lymphocyte. EGFP-fluorescence gradually spread from initially infected neurons to neighboring neurons (Fig. 4B-D). The EGFP-fluorescence in the colonies of neurons has been maintained for at least 6 months. It indicates that EGFP-CDV was well propagated in the infected cells. In any massive EGFP-positive neurons, however typical cell-to-cell fusion and syncytium formation were not found. Focusing on an individual neuron (Fig.

5A-F), infection of EGFP-CDV was observed to spread time-dependently to neighbor neuron via synapses. For example, in Fig. 5B and C, arrow indicates synapitic transmission of one neuron (a) to neighbor (b and c). The transmission of EGFP-CDV was, however, limited to a part of synapses and their related neighbor neurons (Fig. 5D-F, stars), or may be delayed transmission by other part of synapses. To analyze such a selective transmission, the author examined the expression and localization of CDV-membrane proteins (H, F and M) in the neuron (Fig. 6A-C). By immunohistochemistry of the whole hippocampal slices, CDV-H and -F were detected in whole neuron including soma, dendrites and synapses, while CDV-M was detected in mainly soma. In a part of dendrites and synapses when EGFP-fluorescence was detected (Fig. 6C; stars), CDV-M positive synapses seem to be selectively linked to neighbor CDV-infected neurons. Localization of CDV-M in neurons might be different from that of CDV-H or -F, and it seems to be implicated in CDV transmission between neurons.

Electron microscopic analysis of virus assembly and budding in the hippocampal slices

Using electron microscopy (EM), the author compared viral assembly and budding in B95a cells which derived from marmoset B lymphocyte (Fig. 7A-C) and in neurons of the hippocampal slices (7E). Whereas uninfected B95a cells had a smooth cell surface, the surface of CDV-infected cells was highly irregular, with viral particles in different stages of budding, from immature bugles to mature buds (100 to 300nm in diameter) separated from the cell surface. These findings are consistent with previous EM studies of MV infection in Vero cells [Miller, 1979]. In addition, inclusion of RNP was

within the infected cells (Fig. 7E). In the hippocampal slices, presynaptic terminal of CDV-infected neuron also contained inclusion of RNPs. Interestingly, viral particles were located in extracellular space of neuron, likely that viral particles are released to extrasynapic regions (Fig. 7E, VP). This EM may show the budding of CDV at synaptic terminal (Fig. 7E).

DISCUSSION

In this study, author has analyzed CDV infection and transmission manner in dog brain by using the combination of a recombinant EGFP-CDV and dog hippocampal slices. The CDV infection and propagation mainly occurred in CA1-3 regions. Neurons are highly infected with the EGFP-CDV compared to astrocytes and oligodendrocytes. The EGFP-CDV transported from a neuron to the neighbors via synapses. The present data demonstrated that viral envelope proteins are well sorted to dendrites and synapses, and RNPs were accumulated at synapses. The selective transmission may be related with localization of M protein in neural cells.

In the case of the infection of MV in hippocampal neuron, it was not clear how MV spread in a brain of natural host, because mice and rats, which are not natural hosts of MV, are mainly utilized as the animal model of MV [Castro, 1972; Kobune, 1983]. Murine neurons with the expression of CD46 have sensitivity to the infection and propagation of MV [Miyagawa, 1997; Rall, 1997; Oldstone, 1999; Evlashev, 2000]. However, the virus spread through human CD46 in mouse brain, which molecule does not exist in original mouse brain cells, could not be considered to show the natural transmission manner in natural host brain. The EGFP-CDV was inoculated in the dog hippocampal slices by way of dropping and covering on the whole surface of slices in the present study. Since the virus was selectively infected in neurons in CA region of the hippocampus, CDV initially penetrates the neurons by unknown receptors. In addition, it is shown that the receptors may be restricted to express or locate at synaptic terminals, because CDV was observed to be transmitted by neuron-to-neuron contact localized at synapses.

Based on the present data, the envelop glycoproteins F and H are rapidly

spread in the whole cell surface of neurons including synapses, whereas the M proteins were mainly located at soma and partially transported to dendrites and synapses. The viral particles are well released from B95a cells infected with CDV or MV, but the mutant viruses which M gene is mutated or deleted, are with loss of function [Cathomen, 1998]. Since EGFP-CDV does not contain any mutation of M gene and the transportation of M protein to dendrites and synapses are at lower level, specific localization of M proteins in neurons may be its original feature. In epithelial cells infected with MV, the envelope glycoproteins F and H are targeted essentially to the basolateral domain, but M protein retargets to the apical domain [Naim, 2000]. This indicates that the sorting does not always synchronize with the glycoproteins. Our EM analyses suggest that the formation and budding of viral particles occur at synapses. Taken together, these results suggest that the accumulation of M proteins at synapses is necessary for transmission of CDV via synapses and the difference of accumulation at each synapse may cause a selective transmission to neighbor neuron.

The spread of CDV infection requires that RNP is transferred from one infected neuron to another, but it is not clear how the RNP is transported to neighbor neuron and whether the transport is mediated by viral particle. Most of in vitro and in vivo analyses have failed to demonstrate that the viral particles of MV or CDV are released from neurons or neuronal culture cells, and thus the transport of RNP is mainly considered to mediate cell-to-cell contact without virus particle release [MV: McQuaid, 1998, Duprex, 1999, Duprex, 2000, Plumb, 2002; CDV: Zurbriggen, 1995, Meertens, 2003, Plattet, 2004]. Our EM analyses demonstrated that RNP could be found within neural process and accumulated in synapses. Moreover, it is observed that the viral particles are released to extrasynaptic regions. Lawrence and colleagues reported that primary

mouse neurons and human neural cell line NT2 infected with the MV Edmonston strain produced viruses and released to extracellular at even 1,000-fold fewer level than Vero cells, [Lawrence, 2000] and Mcquaid and colleagues visualized a budding of MV in NT2 cells [McQuaid, 1998]. Large dense-core vesicle, which is of approximately 200nm at a diameter and includes neuropeptide and other neurotransmitter, are known to be released from synaptic terminal [Schwab, 1983]. These data suggest that neuron has a minimal ability of formation and budding of the viral particles, and the viral particles are transported to the synapses of neighbor neurons through extrasynaptic regions. The author also hypothesized that the transport of RNP is capable through trans-synaptic route in synaptic cleft. However, since synaptic cleft between synapses is at a distance between 20-50nm, typical viral particles with a diameter of more than 100nm could not be completely released to the synaptic cleft and if virus is budding, the particle immediately fuses to postsynaptic membrane. Therefore it might be very difficult to present such a synaptic fusion mediated by viral particles.

The use of viral vectors to deliver genes to the CNS provides as a great progress for both basic research and therapeutic application. Viral vectors are better in the high expression level of genes comparing with plasmid vectors using lipofection and calcium phosphate methods, and cell type specificity is also more selective than electroporation and gene gun. Our data showed that the EGFP-CDV is favorable for neuronal infection and the expression of EGFP is observed more than 6 months without cytotoxicity and syncytium formation of neurons. This agrees with the studies of MV that using rat hippocampal slices, neurons infected with MV retain normal morphology and physiology by electrophysiological analyses [Ehrengruber, 2001]. Ehrengruber and colleagues reported that Semliki forest virus is useful for short-term and

Adeno-associated virus and Lentivirus for long-term transduction of rat hippocampal slices [Ehrengruber, 2001]. In this study, the author showed that the EGFP-CDV infection in dog hippocampus was initially observed within 24 hrs after infection and green fluorescence has be continuously detectable more than 6 months. CDV has advantages that CDV neither cause integration of the virus gene into host genomes like as Retrovirus nor cause homologous recombination like as Herpesvirus. The results in the present study suggest that CDV is available for long-term transduction of neurons as a delivery vector.

FIGURE LEGENDS

Fig. 1: (A) Schematic diagram of hippocampus. Hippocampus consists of CA1, 2 and 3 regions and dentate gyrus. Glutamate acid neuron were distributing at pyramidal cell layer. (B) EGFP-CDV inoculation method on dog hippocampal slice. 1x 10^5 $TCID_{50}$/ml EGFP-CDV was inoculated by dropping on the surface of the slice.

Fig. 2: (A) Distribution of EGFP-positive cells. (B) Summarized EGFP-positive areas in total 10 slices.

Fig. 3: Distribution of EGFP positive cells in the hippocampal slices. Astrocytes, oligodendrocytes and neurons were determined by anti GFAP, CNP and Map2abc antibodies respectively.

Fig. 4: Spread of EGFP-CDV in neural cells. EGFP were observed in neural cells infected with EGFP-CDV and the EGFP-CDV spread around cells during the observation period. (A) 24 hrs post infection. (B) 5 days post infection (dpi). (C) 11 dpi. (D) 17 dpi.

Fig. 5: EGFP-CDV transmission to neurons by synapses. (A) 4.5 dpi. (B) 5 dpi. (C) 7 dpi. (D) 9 dpi. (E) 13 dpi. (F) 16 dpi. Initially, EGFP-CDV infected a neuron (a) and then transmitted to other neurons (b-e). Arrows indicate the synaptic regions where the virus transmission occurred. Stars indicate synapses which EGFP-CDV was not transmitted.

Fig. 6: Immunohistochemical analysis of EGFP-CDV infected neurons using anti-CDV H (A), F (B) and M (C) proteins. Each protein reacted with each specific monoclonal antibody and then with rhodamin-pharoisine conjugated secondary antibody. H and F proteins were expressed and widely distributed in the infected neurons. M proteins were, however, distributed mainly at soma and at limited dendrites or synaptic regions.

Fig. 7: Electron microscopic analysis. (A-C) The CDV Yanaka strain infected B95a cells. (D) B95a cells. (E and F) EGFP-CDV infected hippocampal cells. Virus particles, budding forms or assembly stage of viruses were observed (A-C). Viruses were released from the surface of unlimited regions. Arrows indicate virus particles. Virus particles existed at the extrasynaptic spaces close to synapse and between neighbor cells (E). The virus particles on the large scale are shown in (F). SV: synaptic vesicle, VP: virus particle, RNP: ribonuclecapsid, ER: endoplasmic reticulum, G: granule, M: mitochondria, cleft: synaptic cleft.

Fig. 1

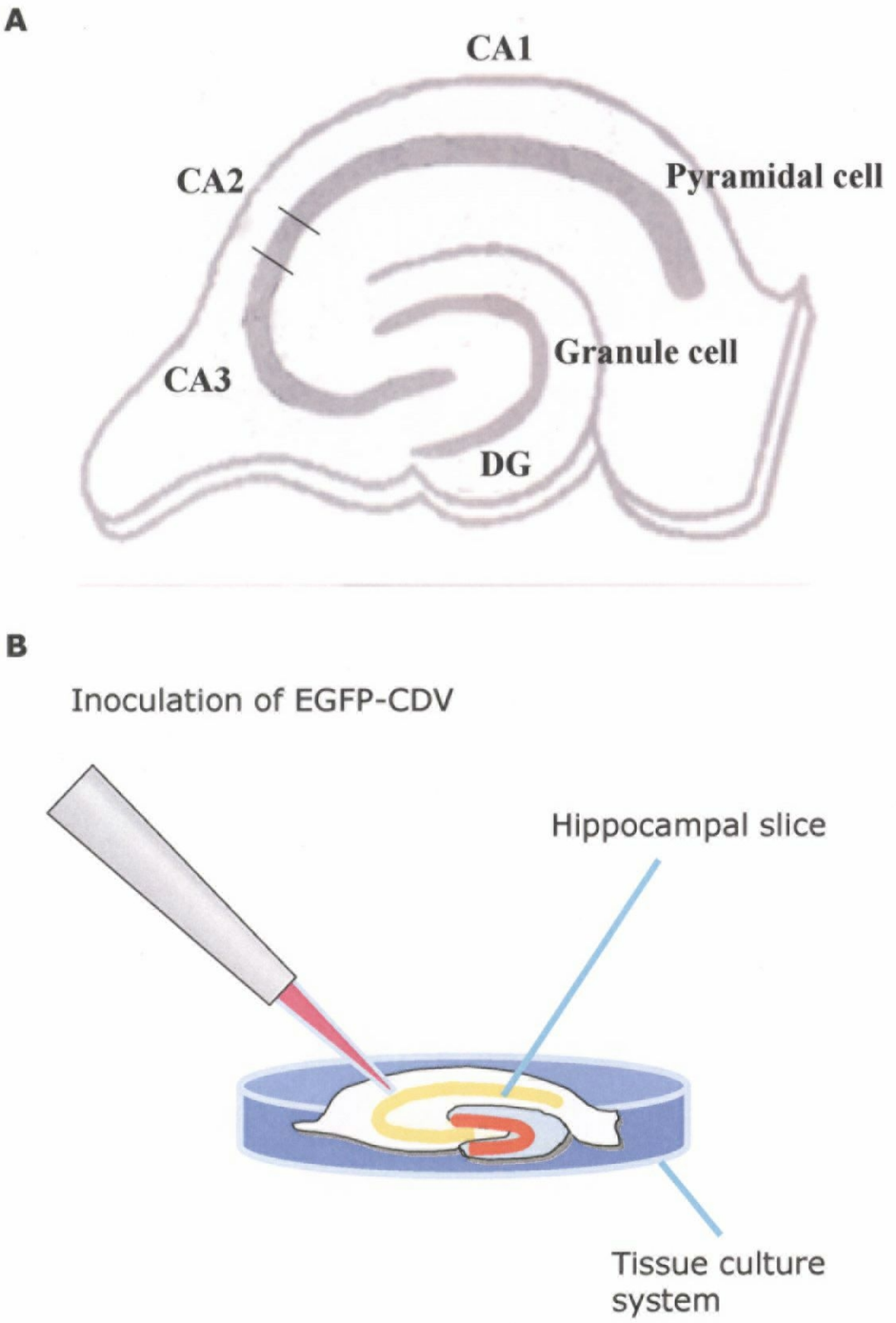
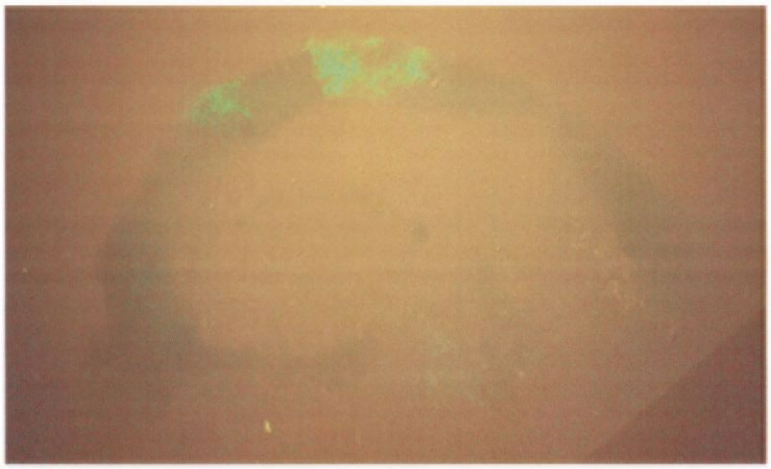

A
CA1
CA2
Pyramidal cell
Granule cell
CA3
DG
B
Inoculation of EGFP-CDV
Hippocampal slice
Tissue culture system

Fig. 2

A



B

C

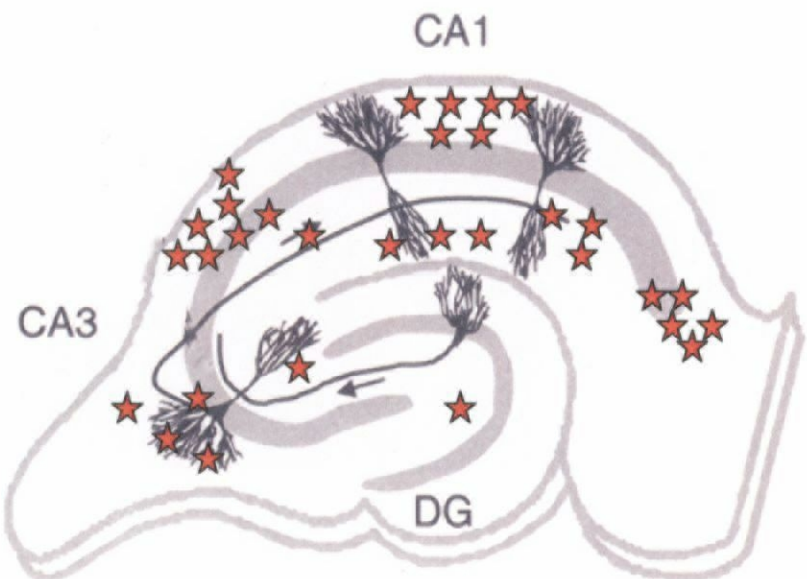


Fig. 3

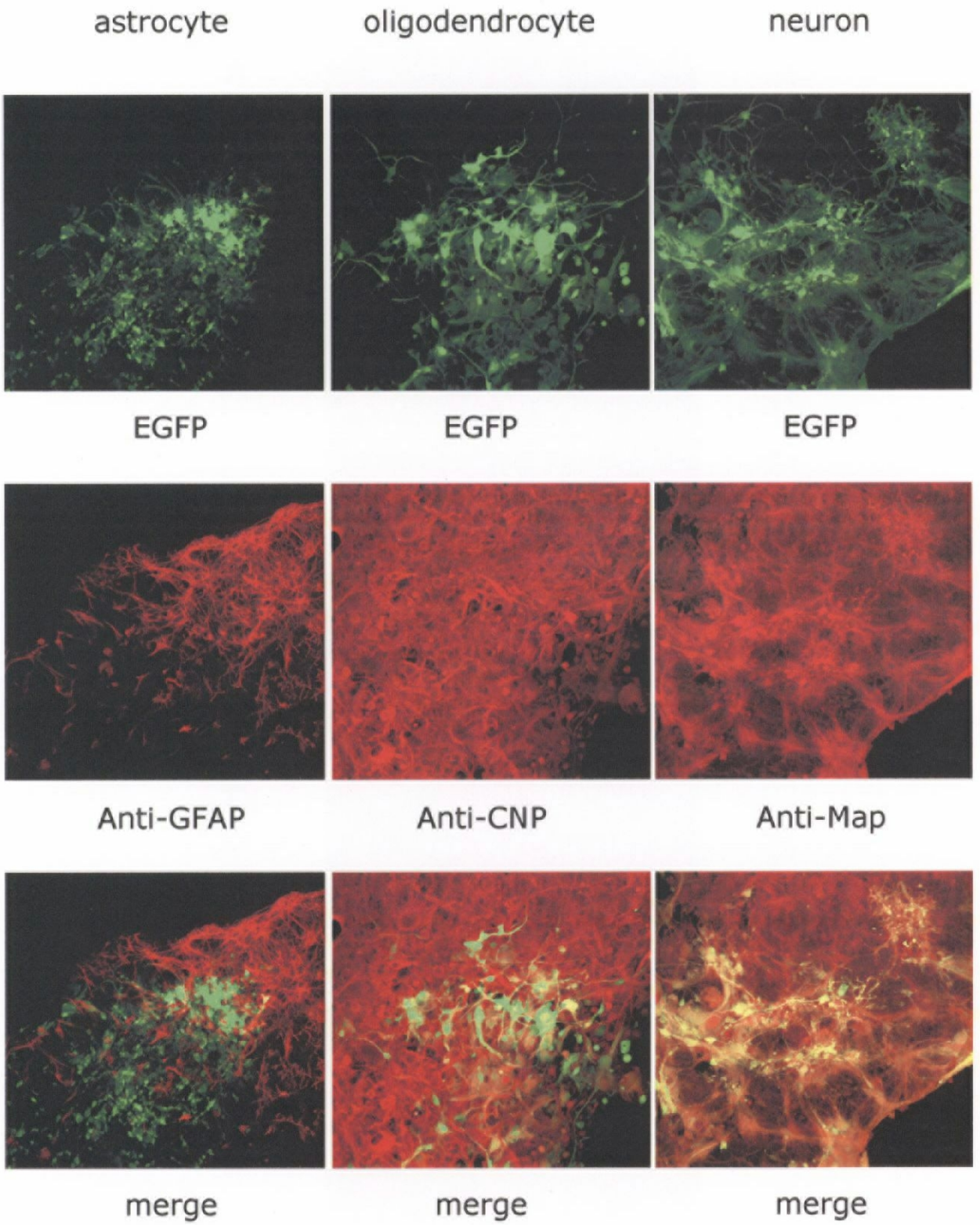

astrocyte
oligodendrocyte
neuron
EGFP
EGFP
EGFP
Anti-GFAP
Anti-CNP
Anti-Map
merge
merge
merge

Fig. 4

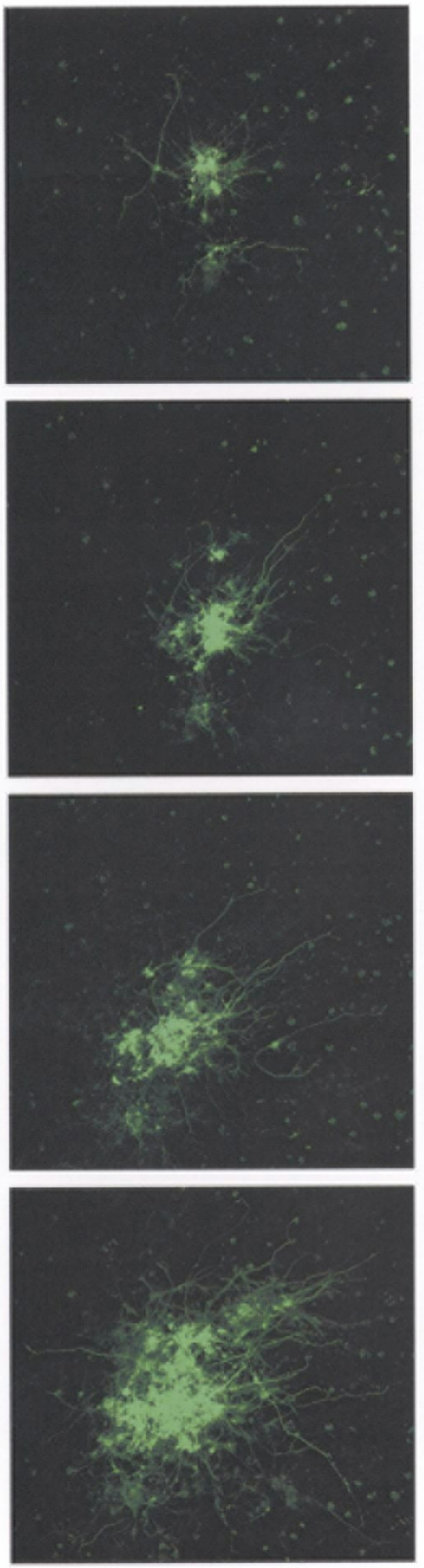


Fig. 5A-C

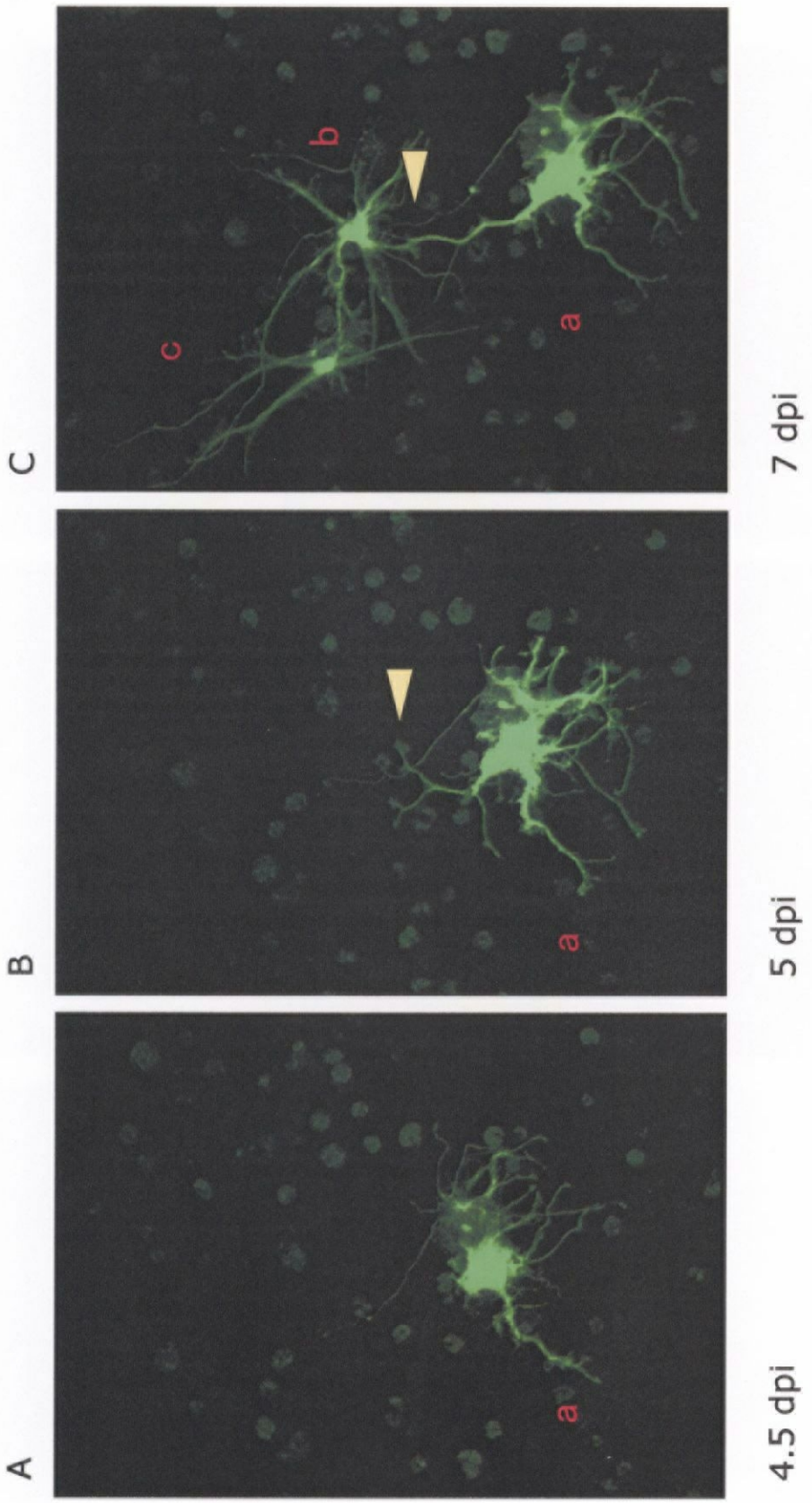


Fig. 5E-F

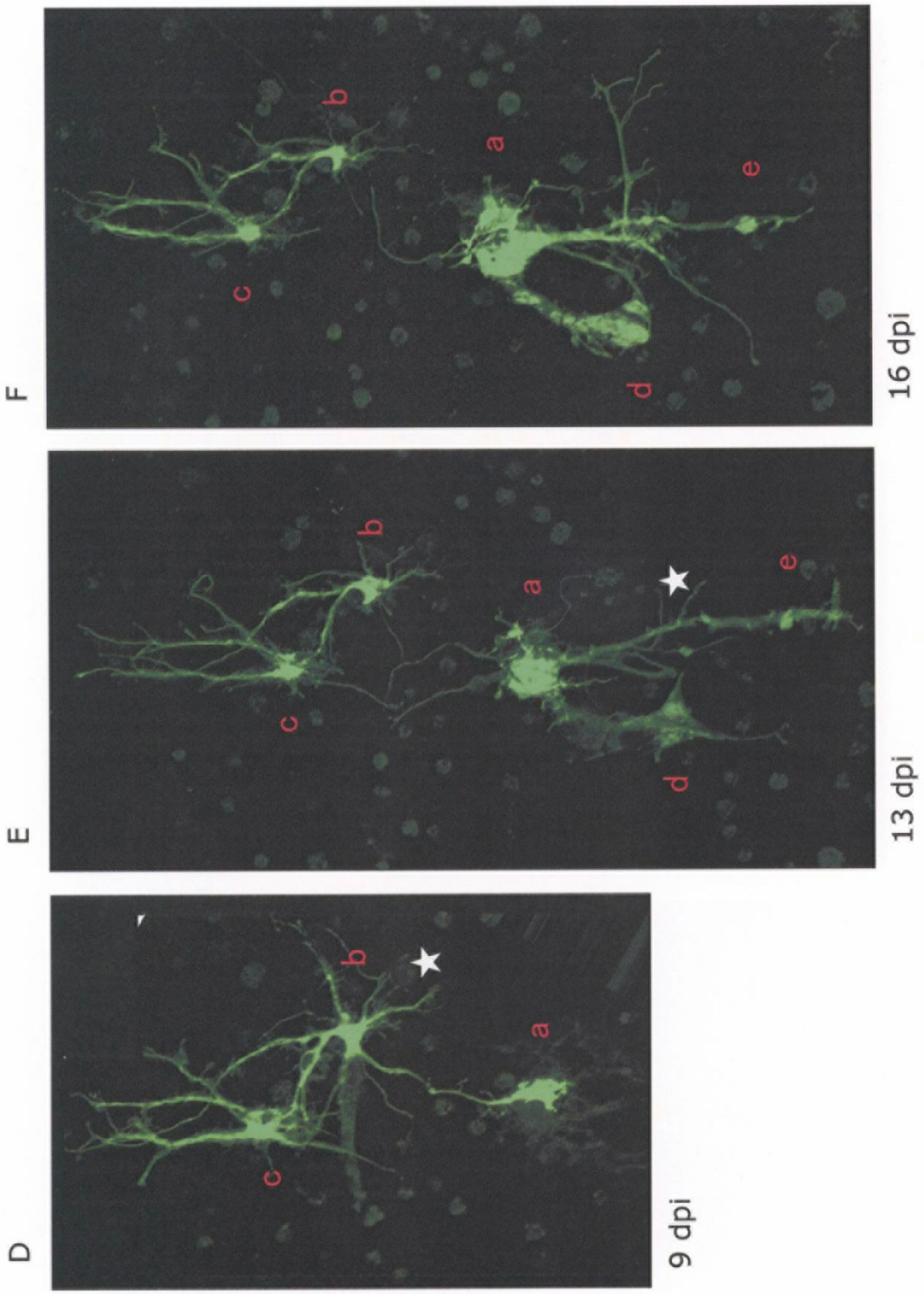


Fig. 6A

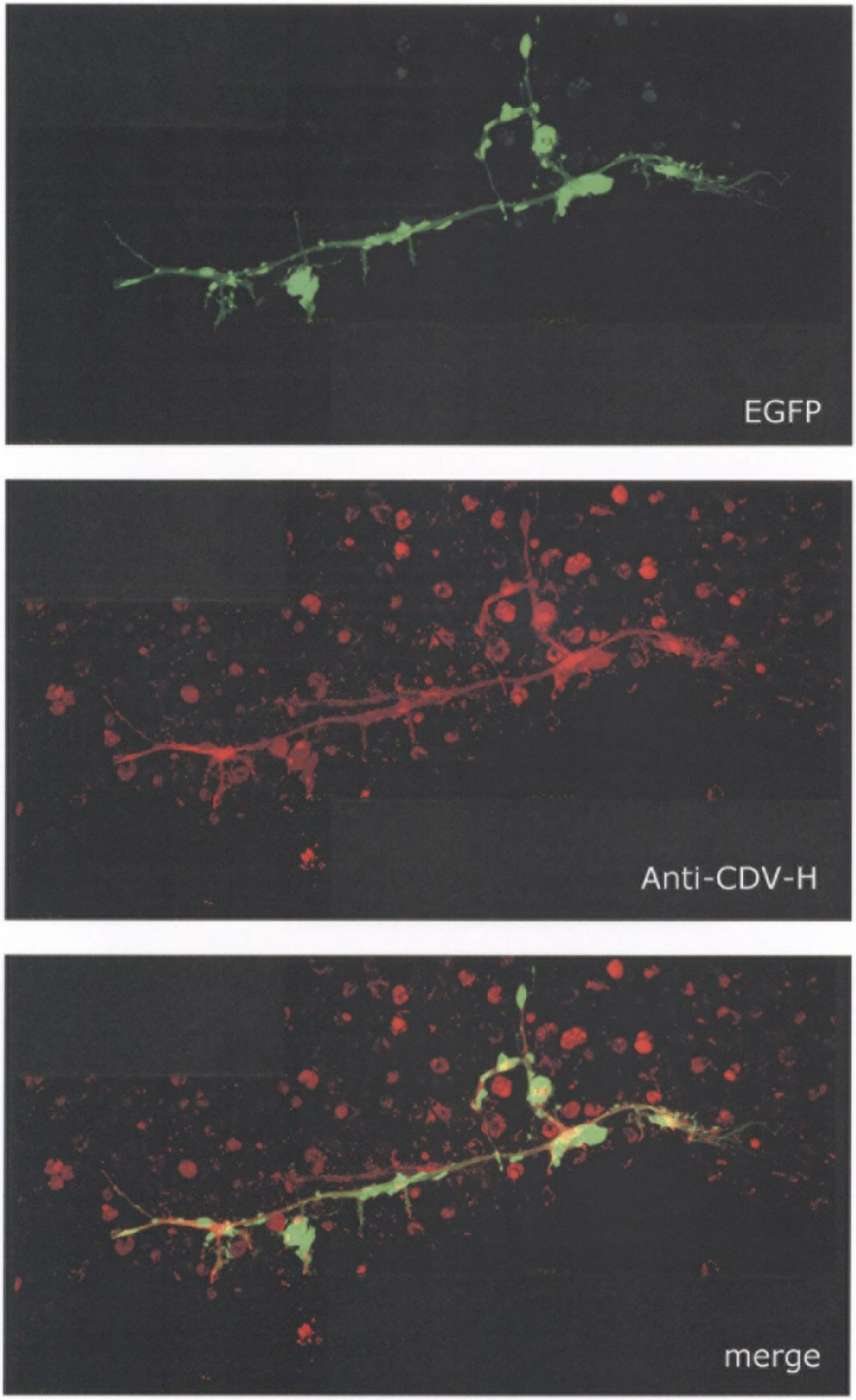
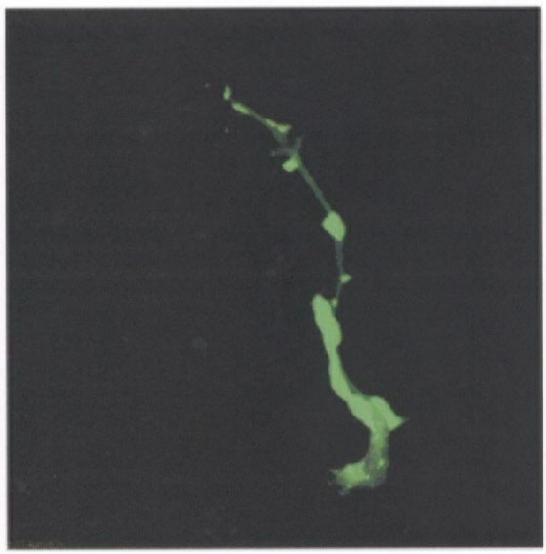
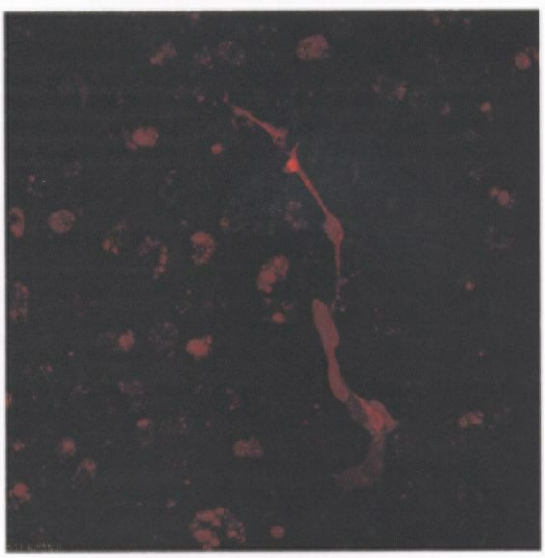


Fig. 6B

EGFP



Anti-CDV-F



merge

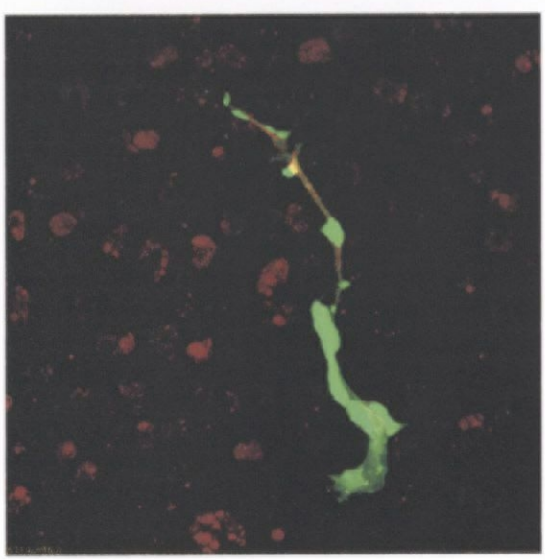


Fig. 6C

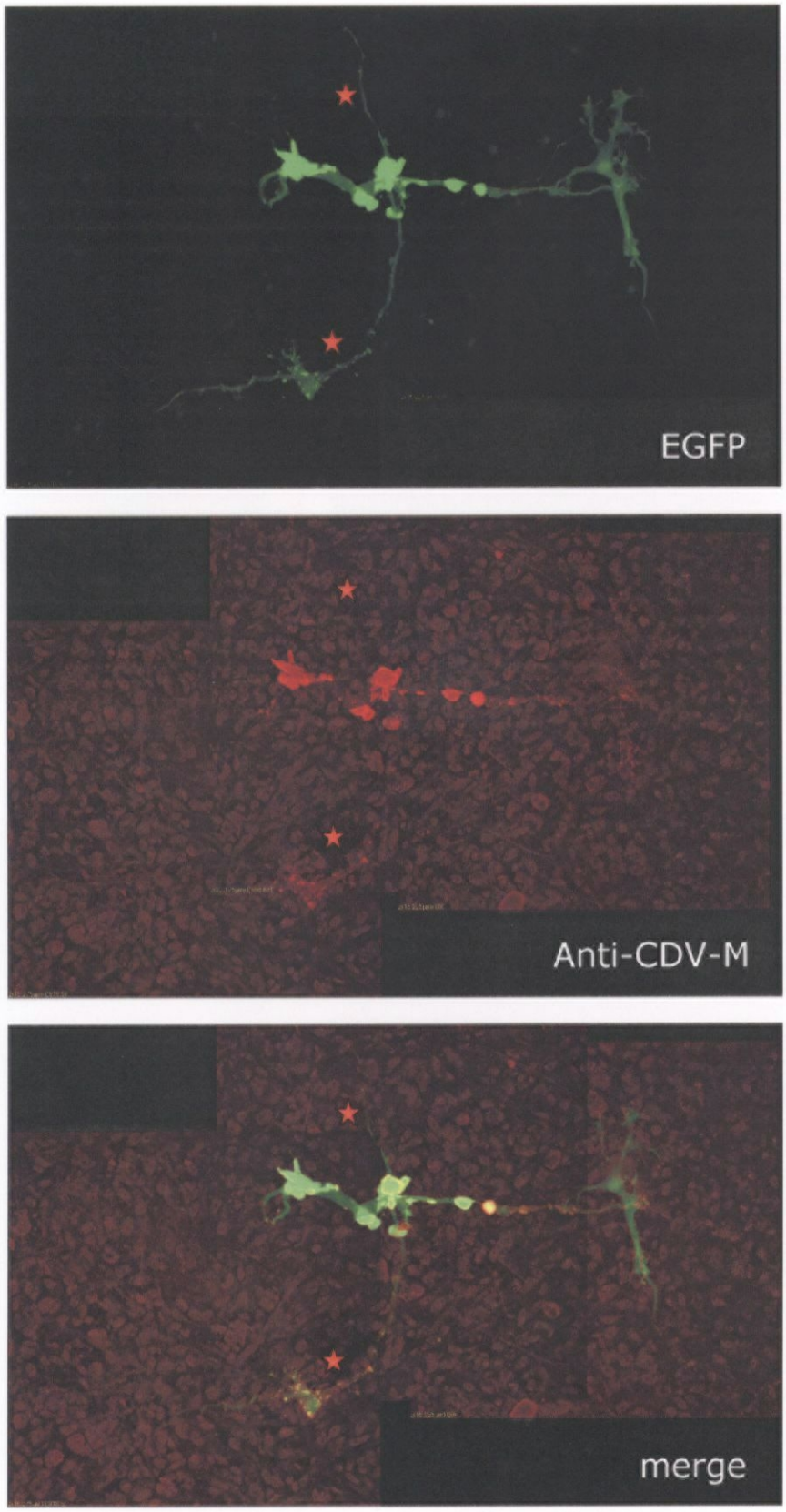


Fig. 7A-D

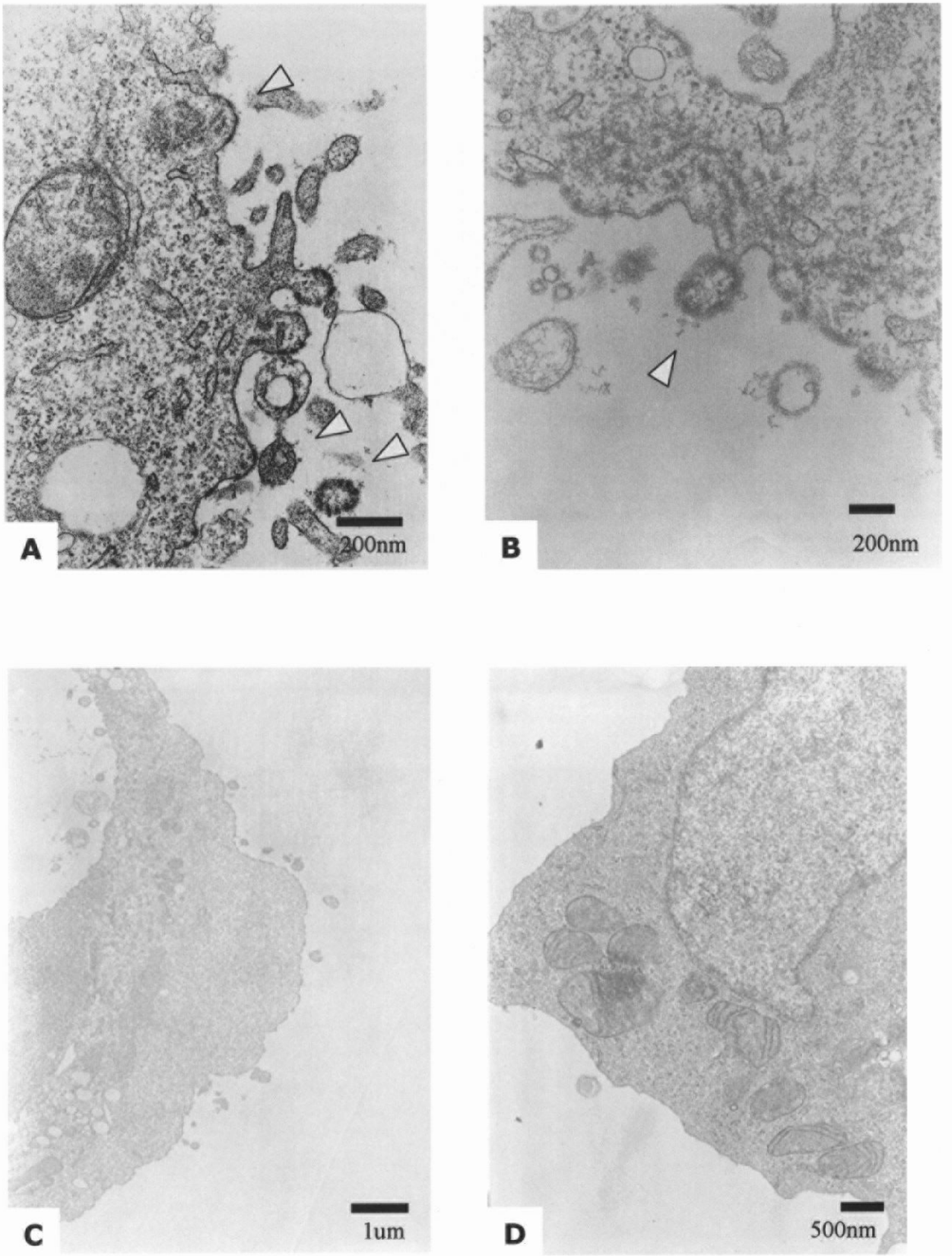


Fig. 7E, F