resuspended in an induction medium (IM), which is composed of DMEM/F-12 supplemented with 1% NCS, 1× Antibiotic-Antimycotic, 1× Glutamax, 10 ng/mL BDNF and the inducing agent taurine (50 μmol/L) plus RA (10 μmol/L), then plated onto poly-D-lysine-coated 8-well culture slides to culture for an additional 8 days (Das *et al.* 2006; Osakada *et al.* 2008). Cells were fixed and immunocytochemistry was performed by staining the retinal photoreceptor markers rhodopsin and recoverin.

Real-time PCR

Real-time PCR was performed as previously described (Sugano *et al.* 2003). Total RNA was isolated from cultured cells using Trizol (Sigma). cDNA synthesis was carried out using the First-Strand cDNA Synthesis kit (GE Healthcare). SYBR Premix Ex Taq (Perfect Real Time; Takara) was used for PCR reactions. Specific transcripts were amplified on a Smart Cycler (Takara) for 35–40 cycles. The expression level of each gene was calculated by normalizing it with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (TaqMan Rodent GAPDH Control Reagents; Applied Biosystems). The primers used in the experiment are shown in Table 1.

Immunocytochemical analysis

Immunocytochemistry was performed by staining cell-specific markers as previously described (Sugano *et al.* 2005; Das *et al.* 2006). Briefly, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, slides were incubated in 1% bovine serum albumin (BSA) and 5% blocking serum for 30 min at room temperature. Primary antibodies were added and incubated overnight at 4°C. The list of antibodies and their dilution are given in Table 2. Slides were washed and incubated with the secondary antibodies conjugated to Alexa Fluor 594 (red) or Alexa Fluor 488 (green) (Invitrogen-Molecular Probes) in the dark for 30 min at room temperature. A negative control was performed by replacing the primary antibody with normal IgG. For staining of nuclei, cells were covered with Vectashield medium including 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories Inc.). Fluorescence was excited and labeled cells were imaged with a fluorescence microscope (Axiovert40; Zeiss, Germany).

Protein extraction and western blotting

Western blotting analysis was performed as previously described (Takahashi & Yamanaka 2006). Briefly, the NIH/3T3 cells were lysed with RIPA buffer supplemented with cocktail (Roche), and cell lysates (50 μg) were separated by electrophoresis on Mini-PROTEAN TGX gel (BIO-RAD) and transferred to an immuno-blot PVDF membrane (BIO-RAD). Antibodies used were Sox2, Nestin (1:200, shown in Table 2), anti-rabbit and anti-mouse IgG (H&L) AP conjugate (1: 7500, Promega).

Statistical analysis

The data of real-time PCR analysis are expressed as mean ± SD. Significance between groups was analyzed by one-way analysis of variance (ANOVA) with GraphPad Prism 4.0 software (San Diego). Values of $P < 0.05$ were considered statistically significant.

Results

NIH/3T3 fibroblasts can form neurosphere-like cells in defined conditions

First, we carried out a neurosphere assay on floating NIH/3T3 cells cultured in two different proliferating media: NSCm and NC. When NIH/3T3 cells were cultured in suspension for 2–5 days, NIH/3T3 cells formed spheres (Fig. 1B,C), which displayed classic features of neurospheres, in both proliferating media. There was no apparent difference in morphology between NC- and NSCm-cultured spheres for the first 2–3 days of culture. All NSCm-cultured spheres had a regular and round shape with bright borders on the edge of spheres (Fig. 1B). However, after 4–5 days of culture, the diameter of NC-cultured spheres did not increase, and some of these spheres showed an irregular and unhealthy appearance with dark or indistinct borders (Fig. 1C), which was assumed to be

Table 1. Sequences of primers used in real-time polymerase chain reaction (PCR)

Gene	Primer sequence (5′-3′) F	R	Product (bp)	Annealing temp. (°C)	GeneBank accession number
Nestin	AGACAGTGAGGCAGATGAGT	ATGAGAGGTCAGAGTCATGG	224	55	NM_016701
Sox2†	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA	296	60	NM_011443

†Primers of Sox2 were from Takahashi & Yamanaka (2006).

Table 2. List of antibodies used to stain different target cells

Antibody	Species	Dilution	Company and catalog no.	Target cells
Nestin	Mouse	1:500	Millipore-Chemicon:MAB353	Neural progenitors
Sox2	Rabbit	1:100	Santa Cruz Biotechnology, Inc: sc-20088	Neural progenitors
Msi1	Rabbit	1:100	Sigma-Aldrich: M3571	Neural progenitors
Pax6	Rabbit	1:100	Santa Cruz Biotechnology, Inc: sc-32766	Neural progenitors
BrdU	Mouse	1:100	Santa Cruz Biotechnology, Inc: sc-32323	Proliferating cells
β-tubulin	Mouse	1:500	Sigma: T5076	Neurons
NF200	Mouse	1:100	Sigma: N0142	Neurons
GFAP	Goat	1:100	Santa Cruz Biotechnology, Inc: sc-6171	Astrocytes
O4	Mouse	1:100	Chemicon International, Inc.: MAB345	Oligodendrocytes
Rhodopsin	Mouse	1:100	Millipore-Chemicon: MAB5316	Photoreceptors
Recoverin	Goat	1:100	Santa Cruz Biotechnology, Inc: sc-20353	Photoreceptors

BrdU, 5-Bromo-2′-deoxyuridine; GFAP, glial fibrillary acicic protein; Msi1, Musashi homologue 1; NF200, neurofilament 200 kDa; O4, oligodendrocyte marker O4; Pax6, paired box protein 6; Sox2, SRY (sex determining region Y)-box containing gene 2.

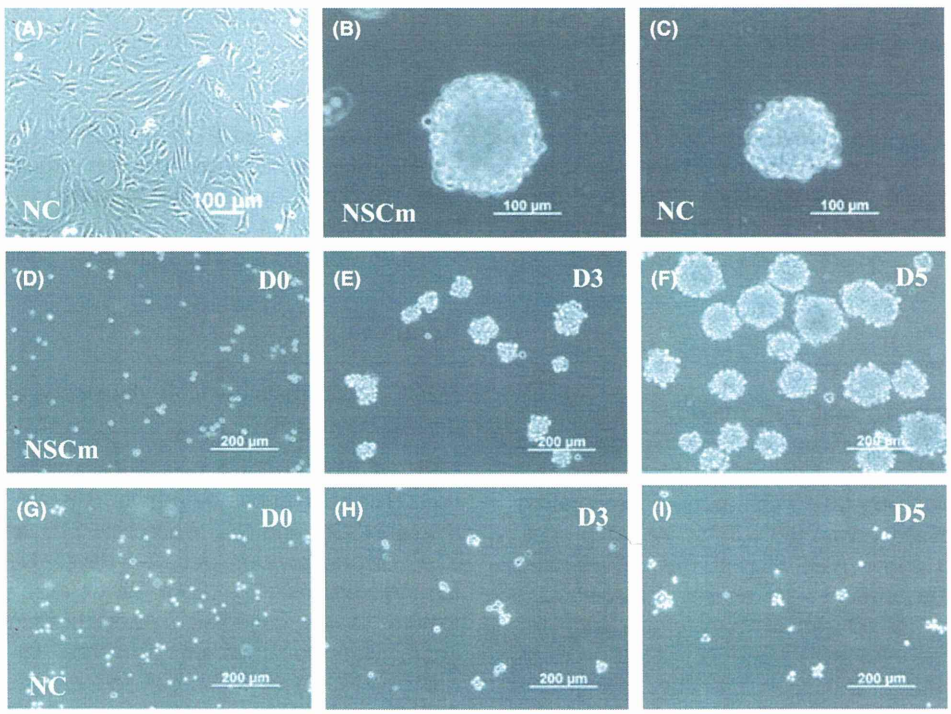

Fig. 1. Generation and passage of NIH/3T3-derived neurosphere-like cells. NIH/3T3 fibroblasts were adherently cultured in normal condition (NC) on normal (uncoated) dishes (A). Spheres were generated after culturing in neural stem cell medium (NSCm) (B) or NC (C) on 2% agarose-coated dishes for 5 days. Generation of the secondary spheres were carried out by culturing in NSCm (D–F) or NC (G–I) for 0, 3, and 5 days. The NSCm-cultured secondary spheres were observed on day 3 (E) after passaging, and the diameter had doubled by day 5 (F). NC-cultured spheres formed very small secondary spheres, and the diameter was unchanged after 3–5 days of culture (H and I).

surrounded by many dying cells caused by the lack of necessary growth factors.

Second, we tested the ability of NIH/3T3-derived spheres to generate secondary spheres. After dissociating into single cells and culturing for 3–7 days, the secondary spheres were quickly formed (on days 3–5) in NSCm, and the sphere size was dependent on culture time with defined cell density (Fig. 1D–F). These cells could generate sub-spheres for an extended period of three passages (more passages were untested). However, NC-cultured spheres formed only very small secondary spheres on days 3–5 after passaging (Fig. 1G–I), and tertiary spheres were difficult to generate.

NIH/3T3-derived spheres express neural progenitor markers

Third, we performed immunocytochemistry to stain the neural progenitor markers Nestin, Sox2, Pax6, and Msi1 for NSCm-cultured NIH/3T3-derived spheres, and the results showed that these cells expressed neural progenitor markers (Fig. 2E–T). Some of these spheres co-expressed Nestin and Sox2 (Fig. 2J–L), suggesting that some cells expressed multiple neural progenitor markers. Double staining for Sox2, Pax6, and Msi1 with BrdU indicated that these spheres were composed of dividing cells that entered the cell cycle (Fig. 2N–P,R,T).

To compare the neural progenitor potential of NIH/3T3 cells cultured in different conditions, the expression of neural progenitor markers Sox2 and Nestin were examined by real-time PCR. Sox2 (Fig. 2V) and Nestin (Fig. 2W) were significantly upregulated in NSCm-cultured spheres compared with adherent NIH/3T3 fibroblasts or NC-cultured spheres. Moreover, the expression of Nestin and Sox2 were also observed from NSCm-cultured spheres by western blotting (Fig. 2X).

NIH/3T3-derived spheres have the potential to differentiate into neuronal cells

Subsequently, we tested whether NIH/3T3-derived spheres can be differentiating into neuronal cells. After transferring to the DM, these spheres were cultured for another 8 days. Immunocytochemical results showed that these cells expressed the neuronal markers β-tubulin (Fig. 3D) and NF200 (Fig. 3H) and the astrocytic marker GFAP (Fig. 3K), although expression of GFAP was very low. However, these cells did not express the oligodendrocyte marker O4 (data not shown).

NIH/3T3-derived spheres can be induced to express retinal photoreceptor markers

Finally, to determine the ability of NIH/3T3 cells to differentiate along neural lineage, we treated NIH/3T3-derived neuron-like cells with taurine and RA, both of which show effective promotion of neuron induction. After treatment with these chemicals, expression of the neuronal marker β-tubulin (Fig. 4E) was greatly enhanced, and expression of photoreceptor markers rhodopsin (Fig. 4I,K,M) and recoverin (Fig. 4L,M) was also induced. Double staining results showed that some cells co-expressed recoverin and rhodospin (Fig. 4K–M); however, the expression of recoverin was very low (Fig. 4L–N). Real-time PCR analysis showed that neural progenitor markers Sox2 and Nestin were significantly downregulated during the differentiation and induction of neuron- and photoreceptor-like cells (Fig. 4O,P).

Discussion

Many studies have shown that the undifferentiated cells, such as ES cells, ES-derived neural stem cells (NSCs), bone marrow stromal cells or iPS cells have the ability to be differentiated along the neuronal lineage (Sanchez-Ramos *et al.* 2000; Woodbury *et al.* 2000; Zhao *et al.* 2002; Ikeda *et al.* 2005; Takahashi & Yamanaka 2006; Osakada *et al.* 2008, 2009; Hirami *et al.* 2009; Jin *et al.* 2009) and could be potential targets for the replacement therapy for retinal degeneration diseases. However, the ability of differentiated cells to be transdifferentiated into neuronal cells has not been widely investigated. Zhang *et al.* (2010) showed that the NIH/3T3 fibroblasts were able to be induced to express neuronal markers after the epigenetic modification by adding epigenetic modifiers, but the question of whether the differentiated cells could be transdifferentiated into neuronal cells without adding any epigenetic modifier and the mechanism involved still remain to be characterized.

Our study showed that NIH/3T3 fibroblasts were able to form spheres composed of dividing cells in suspension culture in the presence of EGF, bFGF and B27 supplement (without vitamin A), which are conditions suitable for the proliferation of neural progenitors. These spheres were able to be serially passaged to form more sub-spheres, and these cells were incorporated with BrdU, indicating their ability to self-renew. NSCm-cultured spheres express neural progenitor markers Nestin, Sox2, Pax6 and Msi1, indicating that these cells may have the potential to proliferate toward neural progenitor lineage. NIH/3T3-derived spheres was able to be differentiated into both neuronal and astrocytic cell types by removing EGF and B27 supplement (without vitamin A) from the medium and substituting them with serum, standard B-27 supplement and BDNF or CNTF, and also have the potential to be induced into photoreceptor-like cells. Taken together, these results suggested that NIH/3T3-derived neurosphere-like cells can undergo self-renewal and differentiation into neuron-like cells without any epigenetic modification, which are properties of neural progenitors, suggesting the possible neuronal lineage of NIH/3T3 fibroblasts.

To test whether the NIH/3T3-derived spheres obtained were neurospheres or neural progenitors, three functional attributes that define neural progenitors (or neural stem cells) must be exhibited. The first

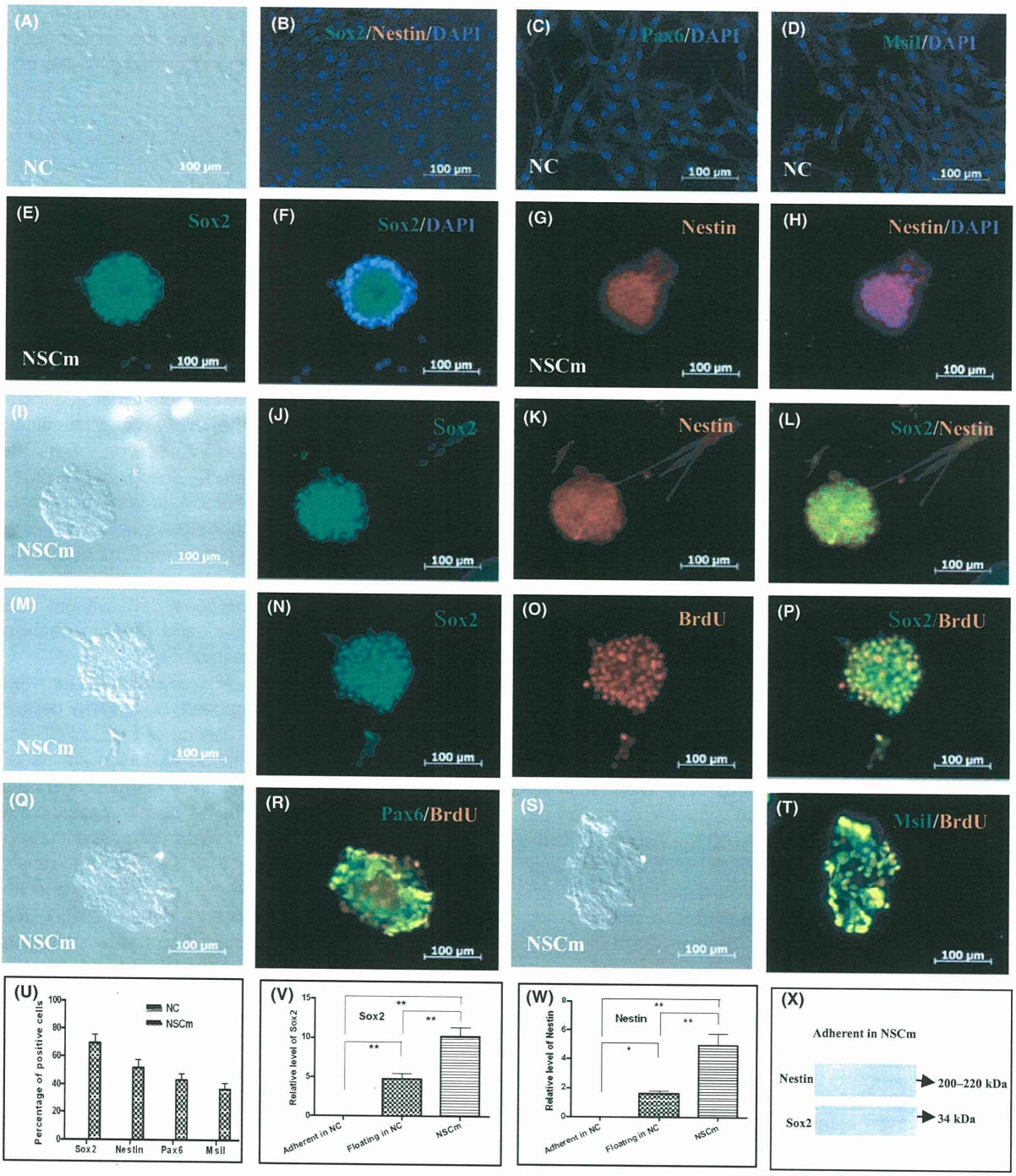

Fig. 2. Neural stem cell medium (NSCm)-cultured NIH/3T3-derived neurosphere-like cells expressed neural progenitor markers. NC-cultured NIH/3T3 fibroblasts did not express any neural progenitor marker (B–D). Single and double staining of Sox2 (E, F, J and L) and Nestin (G, H, K and L) demonstrated that these spheres co-expressed multiple neural progenitor markers. Some spheres were positively stained with BrdU and Sox2 (N-P), Pax-6 (R), Msi1 (T), indicating their proliferative property. Phase contrast images of NIH/3T3 cells cultured in NC (A) and NSCm (I, M, Q and S) were also shown. The percentage of positive cells is presented in the graph (U). Real-time PCR analysis of Sox2 (V) and Nestin (W) were performed for NIH/3T3 cells adherent in NC, floating in NC or NSCm. The columns represent the relative expression level of Sox2 or Nestin in spheres compared with those of adherent NIH/3T3 fibroblasts. Western blotting analysis of Sox2 and Nestin in NC- and NSCm-cultured NIH/3T3 cells were shown in X. The symbols * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

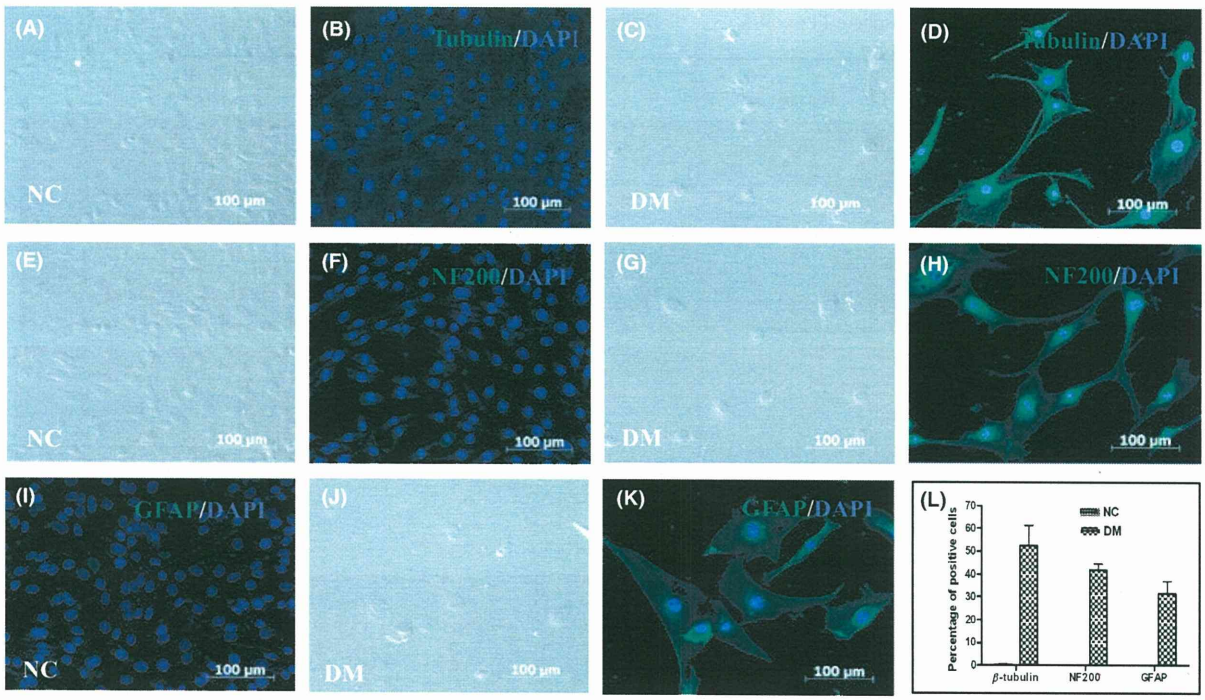

Fig. 3. Differentiation of neural stem cell medium (NSCm)-cultured NIH/3T3-derived neurosphere-like cells into neuron- and astrocyte-like cells. When shifted to DM, these cells expressed markers corresponding to neurons (β-tubulin [D] and NF200 [H]) and astrocytes (glial fibrillary acidic protein [GFAP] [K]). NIH/3T3 fibroblasts cultured in normal conditions (NC) were used as a control (A, B, E, F, and I). Phase contrast images of NIH/3T3 cells cultured in NC (A and E) and DM (C, G and J) were also shown. The percentage of positive cells expressing neuronal or glial markers is presented in the graph (L).

property is self-renewal wherein cells from spheres proliferate and make identical copies of themselves. The second is multipotency, wherein the spheres are able to generate all three main cell lineages of the mammalian central nervous system (CNS), neurons, astrocytes, and oligodendrocytes. The third is the ability to generate tissues. The generation of a neurosphere even from a particular region of the CNS does not necessarily denote to be neural progenitors unless there is supporting *in vivo* evidence (Chojnacki & Weiss 2008; Ahmed 2009). This neurosphere protocol has been used in a number of studies to examine the properties of various progenitors (Chaichana *et al.* 2006; Das *et al.* 2006; Jensen & Parmar 2006; Marshall *et al.* 2006; Chojnacki & Weiss 2008).

In the present study, we demonstrated the self-renewal property of NIH/3T3-derived spheres, and we also showed the potential of these cells to differentiate along two basic CNS lineages, neurons and astrocytes; however, we failed to show the expression of the oligodendrocyte marker O4.

These results predicted two possibilities. One is that these NIH/3T3-derived spheres are not neural progenitors, but only some NIH/3T3 cells with changes in morphology and properties. Because the growth of cells *in vivo* and *in vitro* are tightly regulated by their microenvironments (Hegde *et al.* 2007), NIH/3T3 fibroblasts are likely to survive in NSCm, display classic morphology of neurospheres and respond to growth factor exposure in a similar manner that was exhibited by neural progenitors. For example, the markers of neural progenitors were upregulated and some cells had the potential to differentiate toward neural lineage. However, most of these cells still preserved the property of NIH/3T3 fibroblasts, and could not be differentiated into all three main types of CNS lineages.

The other possibility is that these NIH/3T3-derived spheres may be immature neural progenitors. These spheres could proliferate, express markers of neural progenitors and generate neuron and astrocyte markers. The reason why these spheres did not express the oligodendrocyte marker O4 may be due to the lack of some growth factor(s) in the differentiating medium or the shortage of culture period, which may be critical for the generation of oligodendrocyte progenitors or oligodendrocytes. The cytokine CNTF alone might not be sufficient for the generation of oligodendrocytes, further investigations are needed to detect whether oligodendrocytes can be generated by adding other candidate factor(s), for example, platelet-derived

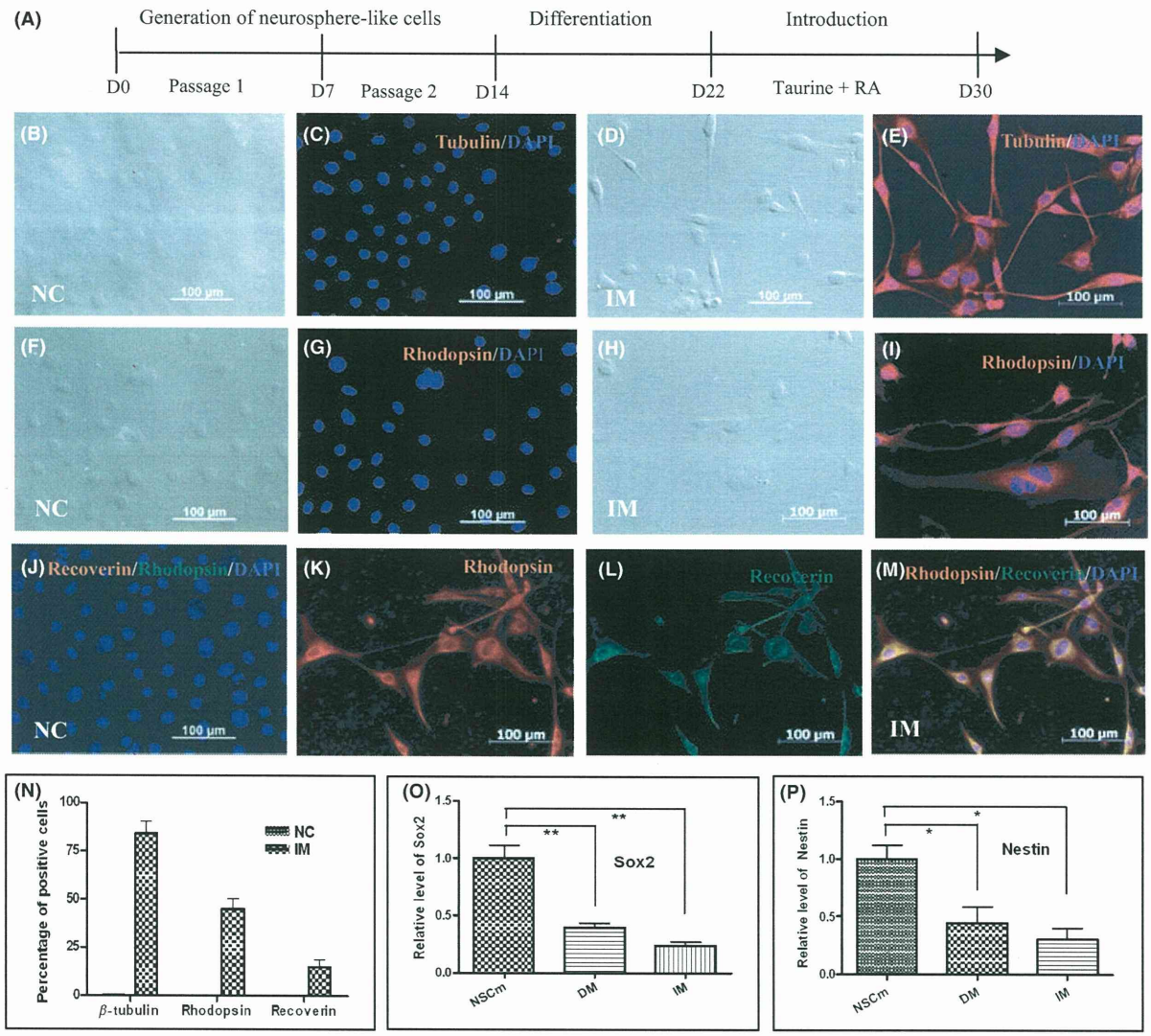

Fig. 4. Induction of neuron- and photoreceptor-like cells by treating cells with the combination of 50 μmol/L taurine and 10 μmol/L retinoic acid (RA). (A). Procedure for induction of retinal photoreceptor-like cells from NIH/3T3 fibroblasts. Immunocytochemical analysis of β-tubulin (C and E), rhodopsin (G, I, J, K and M) and recoverin (J, L and M) was performed for the treated (E, I, and K-M) and untreated (C, G and J) cells. Phase contrast images of NIH/3T3 cells cultured in NC (B and F) and IM (D and H) were also shown. The percentage of positive cells expressing neuronal or photoreceptor markers is presented in the graph (N). Real-time PCR was carried out to analyze Sox2 (O) and Nestin (P) expression in NSCm-, DM- and IM-cultured cells. The symbols * and ** represent $P < 0.05$ and $P < 0.01$, respectively.

growth factor AA, which was demonstrated to effectively enhance survival of oligodendrocyte progenitors (Yang *et al.* 2005; Chen *et al.* 2007).

Our study demonstrates that NIH/3T3 fibroblasts display some features of neural progenitors and express neuron, astrocyte and even photoreceptor markers under defined conditions. These results shed some light on the induction of retinal photoreceptors from a differentiated cell source. Further studies are necessary to determine if NIH/3T3 fibroblasts can be differentiated into functional neurons or photoreceptors, but the present study suggests that neuronal cells can be generated from differentiated cells of other types without the need of adding any epigenetic modifier.

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Evaluation of a Sindbis virus vector displaying an immunoglobulin-binding domain: Antibody-dependent infection of neurons in living mice

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ABSTRACT

Viral vectors that genetically incorporate an immunoglobulin-binding domain on their surfaces provide many advantages because of the availability of a spectrum of antibodies that allow the selection of a wide range of target cells. However, the specificity and the effectiveness of this system have not been evaluated in the field of neuroscience. We investigated the effectiveness and specificity of a recombinant Sindbis virus displaying an antibody-binding domain of bacterial protein A (ZZ Sindbis). We found that the ZZ Sindbis virus vector specifically infected hippocampal neurons in an antibody-specific manner in living mice, although the efficiency of the gene transduction was not high. However, the ZZ Sindbis virus vector that did not display any specific antibodies continued to exhibit intrinsic tropism toward Bergmann glial cells in the cerebellum. These data indicate that the antibody-displaying viral vectors are potentially useful for delivering a gene of interest to a specific subset of neurons in the central nervous system with the help of neuron type-specific antibodies.

1. Introduction

Viral vectors have been powerful research tools for both *in vivo* and *in vitro* studies because of their ease of use and efficient transfection. The cell-targeted expression of a gene is typically accomplished using cell type-specific promoters (Portales-Casamar et al., 2010). This strategy, however, has several disadvantages such as virus toxicity; the promoter is only capable of regulating the expression of the transgene and does not suppress the infection by the viral vector. The expression of the transgene in the target cells may be insufficient because of weak induction by the specific promoter. Moreover, the length of specific promoter regions is sometimes too large to package a viral vector.

Recently, a variety of molecular engineering approaches for modifying the tropism of a virus for a particular cell type have been reported for clinical use in cell-targeting gene therapy (Schaffer et al., 2008; Waehler et al., 2007). Viral vectors that genetically incorporate an immunoglobulin (Ig)-binding domain on their surfaces (Korokhov et al., 2003; Ohno et al., 1997; Ried et al., 2002; Tai et al., 2003) provide a number of advantages because of the availability of a spectrum of antibodies that allow the selection of a wide range of target cells. These viral vectors can selectively infect a specific subset of cells and facilitate the expression of exogenous genes under a variety of regulation strategies (e.g., active promoter or induction mediated by other factors). However, the specificity and the effectiveness of this system have not been evaluated in the field of neuroscience.

In the present study, we investigated the effectiveness and specificity of a recombinant Sindbis virus that displayed an IgG-binding domain of bacterial protein A (Ohno et al., 1997). We showed that this recombinant Sindbis virus vector specifically infected a subtype of hippocampal neurons in an antibody-specific manner in living mice, although the efficiency of the gene transduction was not high. Our results suggest that viral vectors incorporating an IgG-binding domain can be used to deliver a gene of interest to a specific subset of neurons in the central nervous system (CNS) with the help of neuron type-specific antibodies. However, this viral vector that did not display any specific antibodies continued to exhibit intrinsic tropism toward some cells, such as Bergmann glial cells in the cerebellum.

2. Materials and methods

2.1. Cell culture

Baby hamster kidney (BHK) cells were grown in OptiPRO SFM medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 200 mM L-glutamine (Sigma–Aldrich, St. Louis, MO). PC12 cells

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were cultured in RPMI-1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel) and antibiotics (penicillin and streptomycin; Invitrogen Life Technologies).

2.2. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee and were conducted in accordance with the Guidelines for Animal Experiments and Related Activities in Tohoku University and with the guiding principles of the Physiological Society of Japan and the NIH.

2.3. Plasmid construction

The plasmid ZZ SINDBIS m168 (Morizono and Chen, 2005; Morizono et al., 2005) was kindly provided by Dr. Irvin S.Y. Chen (UCLA AIDS Institute, Los Angeles, CA). ZZ SINDBIS m168 was the envelope plasmid for the production of the pseudotyped lentiviral vector and contained a ZZ domain derived from the IgG-binding domain of protein A (Nilsson et al., 1987) that was inserted into the modified E2 domain of the Sindbis virus. This modified envelope gene was transferred into a DH-BB helper plasmid (Bredenbeek et al., 1993) to produce a recombinant Sindbis virus. Briefly, the modified E2 envelope gene containing a ZZ domain and the inverse fragment of the DH-BB gene were amplified by polymerase chain reaction (PCR) using ZZ SINDBIS m168 and DH-BB as templates, respectively. The oligonucleotides 5′-GCACCACTAGTCACGGCAATGTGTTTGC-3′ and 5′-TCACGGCGCGCTTTACAGGCACATAACACT-3′ were used as primers for the modified E2 domain, and 5′-GTAAAGCGCGCCGTGAGTGCCTGACGCC-3′ and 5′-ATTGCCGTGACTAGTGGTGCTGCGGA-3′ were used as primers for the inverse fragment. Each primer contained a unique restriction enzyme BssHII or SpeI site. The resulting PCR products were digested with BssHII and SpeI and were ligated to each other. They were then transformed into Competent High-DH5α (Toyobo Co., Osaka, Japan). The nucleotide sequence of the modified E2 domain was confirmed by DNA sequencing, and the DH-BB-ZZ m168 was obtained.

2.4. Recombinant Sindbis pseudovirion production

Expression vectors harboring the fluorescent protein Venus (Nagai et al., 2002) or mCherry (Shaner et al., 2004), which were named pSinRep5-Venus or pSinRep5-mCherry (Ishizuka et al., 2006), respectively, were used for production of the recombinant Sindbis pseudovirions. RNAs were transcribed from pSinRep5-Venus or pSinRep5-mCherry and DH-BB-ZZ m168 DNA using MEGAscript SP6 Kit (Ambion, Austin, TX). BHK cells were electroporated with these RNAs and were grown for 10 h at 37 °C under 5% CO_2 in MEM alpha medium (Invitrogen Life Technologies) that contained 5% FBS. The cells were then incubated in OptiPRO SFM medium supplemented with 200 mM L-glutamine without FBS. After 24 h, the culture supernatant was harvested, and aliquots were stored at −80 °C. Likewise, the control recombinant Sindbis pseudovirions were generated using the plasmids pSinRep5-Venus and DH-BB. In this way, we produced three types of Sindbis pseudovirions, which were designated ZZ Sindbis-Venus, ZZ Sindbis-mCherry, and DH-BB-Venus.

2.5. In vitro infection assay

To quantify the infection titers of ZZ Sindbis-Venus in the presence or absence of the antibody, rat pheochromocytoma PC12 cells were used as the target cells. The viral solution was mixed with or without the antibody against the extracellular domain of $p75^{NTR}$ (ANT-007; Alomone Labs, Jerusalem, Israel), which is one of the low affinity nerve growth factor receptors and is specifically expressed on the membrane of PC12 cells. After 1 h of incubation at 37 °C, 400 μl of the 10-fold serial dilution of the mixture was added to 50% confluent PC12 cells on 6-well plates. After 1 h of incubation at 37 °C under 5% CO_2, 2 ml of RPMI-1640 with 1% FBS was added to each well. At 24 h postinfection, the number of cells expressing the fluorescence protein was counted using a conventional fluorescent microscopy (Axiovert 200, Carl Zeiss, Göttingen, Germany).

2.6. In vivo infection assay

We used the transgenic mouse TV-42, which specifically expressed synaptopHluorin in a restricted region of the hippocampus (Araki et al., 2005), for targeting. Two microliters of the viral solution containing ZZ Sindbis-mCherry with the GFP antibody (a generous gift from Drs. T. Kaneko and K. Nakamura, Kyoto University, Japan) was stereotaxically injected into the dentate hilus of the TV-42 mice. As a negative control, ZZ Sindbis with GFP antibody was injected into wild-type C57BL/6J mice. The injections of ZZ Sindbis-Venus into the cerebellum were performed on the wild-type mice. For all the injection experiments, the mice (4–6 weeks old, 13–17 g BW) were anesthetized via an intraperitoneal injection of a ketamine–xylazine mixture (50 mg/kg BW ketamine, Daiichi Sankyo Co. Ltd., Tokyo, Japan, and 10 mg/kg BW xylazine, Sigma–Aldrich, St. Louis, MO, USA). Two days after the injection, the mice were ether-anesthetized and decapitated, and the whole brains were quickly removed. The removed brain was rapidly immersed in ice-cold ethanol for 60 min and then in ice-cold methanol for 40 min. The dehydrated brain was embedded in 2.5% agarose gel, and coronal brain slices 250–300 μm in thickness were prepared using a vibratome (Leica, VT1000s, Wetzlar, Germany). The brain slices were observed under an inverted fluorescent microscope (Axiovert 200, Carl Zeiss).

2.7. Immunohistochemistry

The coronal brain slices were fixed at room temperature with 4% paraformaldehyde (PFA) in PBS (0.1 M, pH 7.4) for 30 min, blocked with 100% BlockingOne (Nacalai Tesque, Kyoto, Japan), and treated overnight at 4 °C in PBS containing 5% BlockingOne and 0.1% Triton X-100 with three antibodies: rat monoclonal anti-GFP (1:2000; Nacalai Tesque; cross-reactive to pHluorin), rabbit polyclonal anti-DsRed (1:2000; Clontech, Palo Alto, CA, USA; cross-reactive to mCherry) and mouse monoclonal anti-NeuN (1:2000; Chemicon, Temecula, CA, USA; marker for mature neuron). After washing four times at room temperature in PBS with 0.1% Triton X-100, the slices were treated with secondary antibodies in PBS containing 5% BlockingOne and 0.1% Triton X-100 for 3 h. Alexa Fluor 488-conjugated goat anti-rat IgG (1:200; Invitrogen), Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:200; Invitrogen), and Alexa Fluor 633-conjugated goat anti-mouse IgG (1:200; Invitrogen) were used as secondary antibodies. Finally, the slices were washed four times in PBS with 0.1% Triton X-100 at room temperature and were mounted on glass slides with Permafluor (Thermo Fisher Scientific, Waltham, MA). Each specimen was examined under a confocal laser scanning microscopy (LSM510META, Carl Zeiss).

3. Results

3.1. In vitro infection assay

The p75 neurotrophin receptor ($p75^{NTR}$) is a member of the tumor necrosis factor receptor superfamily and plays many roles in

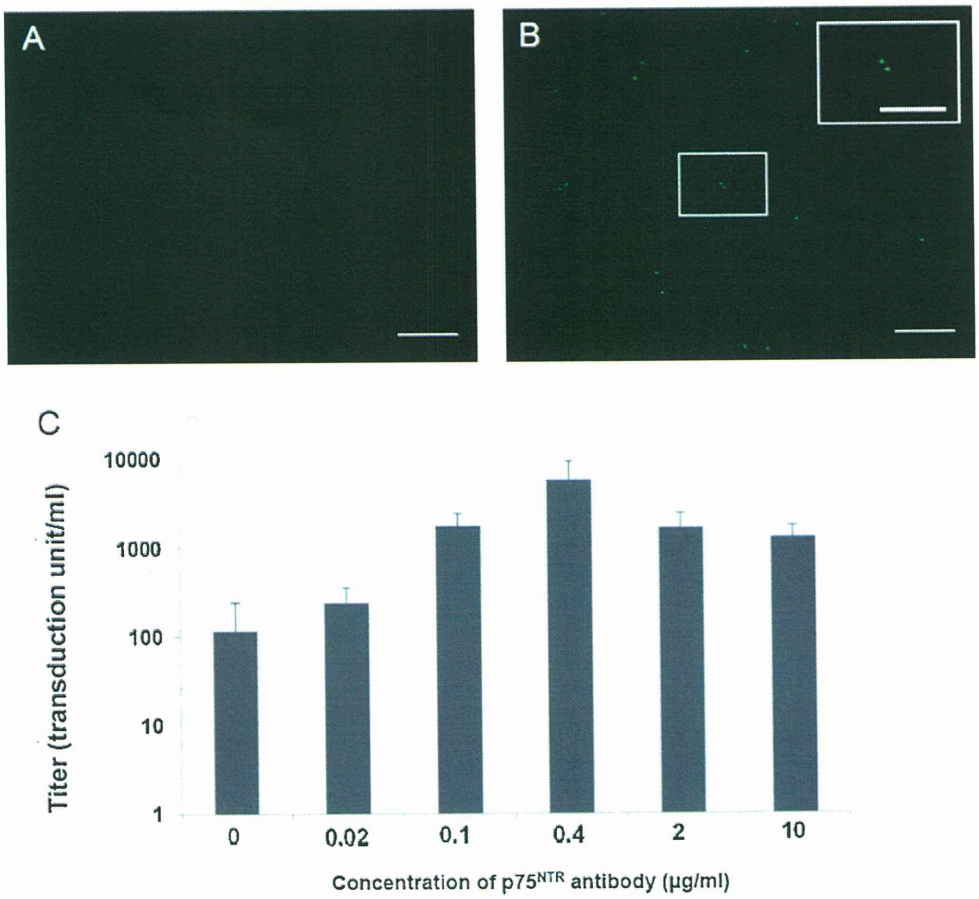

Fig. 1. ZZ Sindbis-Venus with $p75^{NTR}$ antibody infected PC12 cells. (A) PC12 cells treated with ZZ Sindbis without the antibody (bar: 500 μm). (B) PC12 cells treated with ZZ Sindbis with 0.4 μg/ml of the antibody (bar: 500 μm). The inset shows a magnified image of a Venus-expressing cell (bar: 250 μm). (C) Antibody-dependent infectivity titers of ZZ Sindbis-Venus, which is a viral vector displaying immunoglobulin-binding domains. To investigate the dependency of ZZ Sindbis on the concentration of the antibody, PC12 cells were treated with ZZ Sindbis-Venus mixed with various concentrations of $p75^{NTR}$ antibody (p75 Ab), which is shown as the weight of the antibody (μg) per virus solution volume (ml). Titers were calculated by counting the number of cells that expressed Venus. The values shown were obtained from three independent experiments, and the error bars represent standard deviations.

the differentiation, apoptosis, modulation of axonal elongation and synaptic plasticity in the nervous system (Chao, 2003; Dechant and Barde, 2002; Kaplan and Miller, 2000). As each PC12 cell expresses $p75^{NTR}$ molecules in its plasma membrane (Niederhauser et al., 2000), it can be expected that the cells may be transfected with viral genes via ZZ Sindbis pseudovirion vectors in the presence, but not in the absence, of anti-$p75^{NTR}$ antibodies that target the extracellular domain. This was examined using ZZ Sindbis pseudovirions encoding Venus, which is one of the GFP derivatives. As shown in Fig. 1A, the expression of this marker was negligible in the absence of anti-$p75^{NTR}$ antibodies. However, two to three hundred PC12 cells treated with a tenfold diluted virus solution expressed the marker in the presence of the antibody at a concentration of 0.4 μg/ml (Fig. 1B). We then calculated the infectivity titers of the recombinant Sindbis pseudovirions for various concentrations of $p75^{NTR}$ antibodies, taking the number of cells that expressed fluorescent protein Venus as a marker (Fig. 1C). Although the highest percentage of transduced cells was less than 1% and gene transduction was very inefficient, the infectivity titer of ZZ Sindbis-Venus was dependent on the concentration of the antibody, with a positive relationship at lower antibody concentrations and a negative relationship at higher concentrations. This result was likely due to an excess of antibodies binding to the $p75^{NTR}$ and thus competitively inhibiting the antibody-displaying ZZ Sindbis pseudovirions from targeting the molecule.

3.2. *In vivo infection assay in hippocampus*

Synaptobrevin/VAMP-2 is one of the vesicular membrane proteins of small synaptic vesicles in the presynaptic terminal and is involved in docking/priming during exocytosis as one of the SNARE complex elements (Horikawa et al., 1993; Söllner et al., 1993). When this protein is fused with a pH-sensitive derivative of green fluorescent protein (pHluorin) at its intra-lumenal C-terminal, the fusion protein (syanptopHluorin, SpH) increases its fluorescence with vesicular exocytosis and decreases fluorescence with endocytosis and the subsequent reacidification of the intravesicular space (Miesenböck et al., 1998; Yuste et al., 2000; Sankaranarayanan et al., 2000). We recently described the mouse line TV-42, which selectively expressed SpH in the presynaptic boutons of dentate granule cells and the CA1 pyramidal cells of the hippocampus (Araki et al., 2005). As some of the SpH molecules are also distributed in the plasma membrane (Sankaranarayanan et al., 2000; Araki et al., 2005), they can be expected to react with anti-GFP IgGs in the extracellular space.

When the ZZ Sindbis-mCherry was injected into the hippocampus of TV-42 mice with anti-GFP IgGs, red fluorescence was observed in the granule cells of the dentate gyrus (Fig. 2A) and in the pyramidal cells of the hippocampal CA1 region (Fig. 2B). The number of mCherry-expressing neurons varied from trial to trial in our *in vivo* transduction, which was likely due to uncontrollable

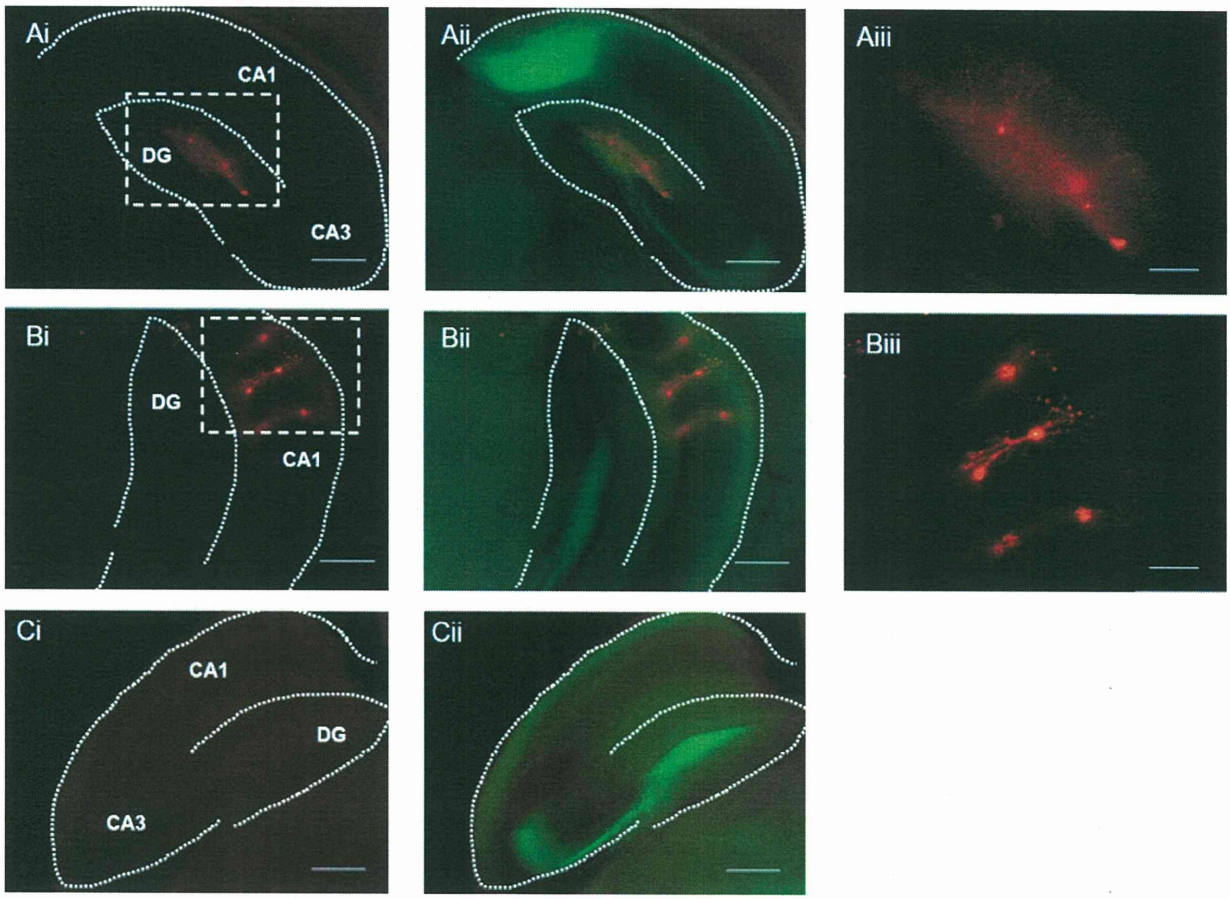

Fig. 2. Fluorescent microscopic images of sliced TV-42 hippocampus that was injected with ZZ Sindbis encoding mCherry. Green or red fluorescence shows either the expression of pHluorin or mCherry, respectively. (Ai–iii) ZZ Sindbis-mCherry with GFP antibody infected in the dentate gyrus. (Bi–iii) ZZ Sindbis-mCherry with GFP antibody infected in the CA1 region. (Ci and ii) ZZ Sindbis-mCherry without antibody. Magnified images of the areas enclosed by the dotted lines in Ai and Bi are indicated in Aiii and Biii, respectively. DG, dentate gyrus. Scale bars: 500 μm (Ai and ii, Bi and ii, Ci and ii); 100 μm (Aiii and Biii).

factors such as the precise site of injection and the diffusion of the viral solution, but it ranged from 2 to 20 neurons in the hippocampus of 6 trials. ZZ Sindbis-mCherry apparently targeted these cells through the binding of the anti-GFP antibody that was displayed on the surface of the viral vector to pHluorin that was expressed in the extracellular surface of the plasma membrane. In contrast, without the antibody, no cells in the hippocampus of the TV-42 mice expressed mCherry (Fig. 2C). Similarly, the mCherry-fluorescence was not detectable in any hippocampal cells of the wild-type C57BL/6J mice, even two days after the injection of ZZ Sindbis-mCherry with the anti-GFP antibody (data not shown).

The ZZ Sindbis-mediated expression of mCherry was also examined in detail using DsRed immunohistochemistry (Fig. 3A). The mCherry-expressing cell shown in Fig. 3A was judged as a CA1 pyramidal neuron of the hippocampus by its position and shape (magnified image in Supplementary Fig. S1). We also detected granule cells of the dentate gyrus using the same immunohistochemical method (Fig. 3B). However, no other types of cells, such as dentate hilar neurons, CA3 neurons or GABAergic interneurons, were identified as positive for mCherry even after immunohistochemistry. The expression of mCherry was also negligible in cells from non-neuronal populations.

3.3. *In vivo infection assay in the cerebellum*

The specificity of the ZZ Sindbis pseudovirion was also tested in the cerebellum. However, the pseudovirion was found to exhibit some tropism toward Bergmann glial cells even in the absence of all antibodies (Fig. 4A). The control DH-BB Sindbis virus also showed strong tropism toward Bergmann glial cells (Fig. 4B). Even at low titers of viral vectors, a number of Bergmann glial cells expressed Venus fluorescence with negligible expression in the neuronal population.

4. Discussion

In the present study, we used ZZ Sindbis, a viral vector engineered to display an IgG-binding domain of protein A, to evaluate the specificity and effectiveness of antibody-displaying viral vectors in the CNS. As these vectors are capable of binding to the target cells by utilizing only antibodies for their cell-surface molecules, it was considered possible for the vectors to deliver an intended gene to a certain subset of neurons in an antibody-specific manner (Fig. 5). Consistent with this expectation, ZZ Sindbis was found capable of cell-specific infection in two combination systems of antibody and target: anti-$p75^{NTR}$ antibody and PC12 cells, which is a well-established model for sympathetic neurons (Fig. 1), and an anti-GFP antibody and TV-42 transgenic mice (Figs. 2 and 3). In particular, the infected neurons in the hippocampus of TV-42 mice were limited to pyramidal neurons in the CA1 and granule cells in the dentate gyrus. Because pHluorin molecules are exclusively expressed in these hippocampal neurons of TV-42 mice (Fig. 3; Araki et al., 2005), the above observation is consistent with the

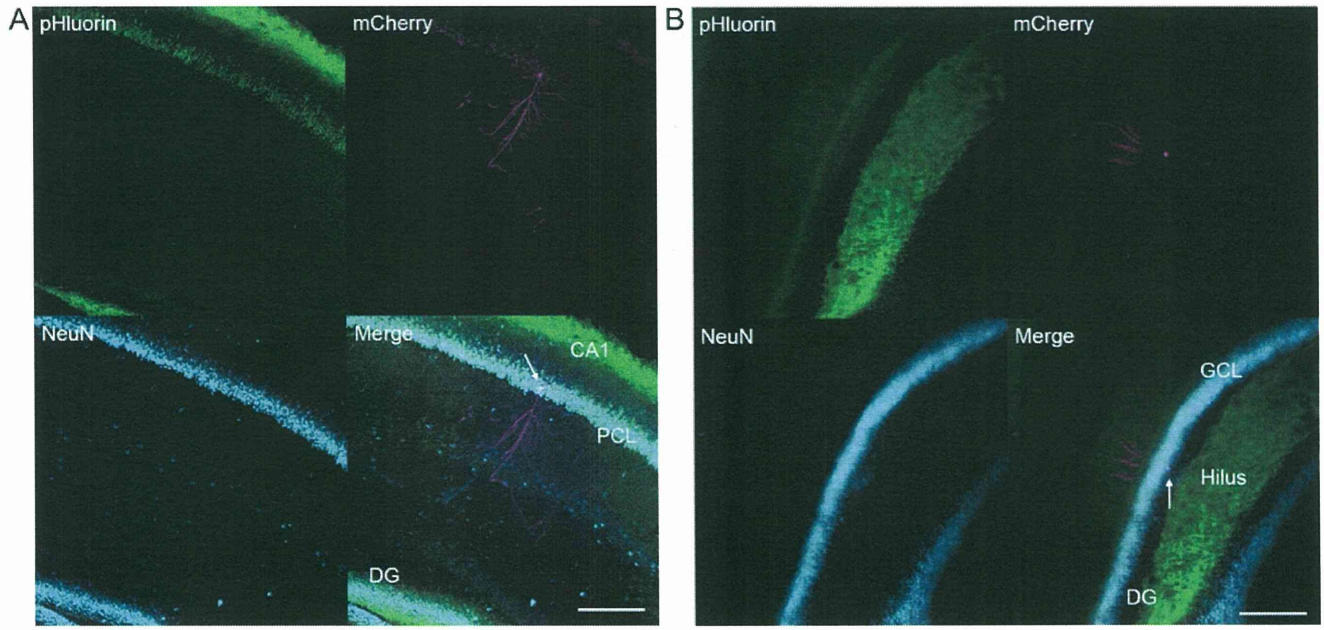

Fig. 3. Antibody-dependent infection of ZZ Sindbis in the hippocampus of a TV-42 transgenic mouse. A neuron expressing mCherry was identified immunohistochemically by anti-GFP (pHluorin), anti-DsRed (mCherry) and anti-NeuN (NeuN), a marker of the neuronal soma. (A) CA1 region. Note that some CA1 pyramidal cells and their presynaptic boutons were reactive to anti-GFP because of the expression of synaptopHluorin. The arrow indicates a typical CA1 pyramidal cell. (B) Dentate gyrus. Some presynaptic boutons in the hilus are reactive to anti-GFP because of the expression of synaptopHluorin. The arrow indicates a typical granule cell. DG, dentate gyrus; PCL, pyramidal cell layer; GCL, granule cell layer. Bar: 200 μm.

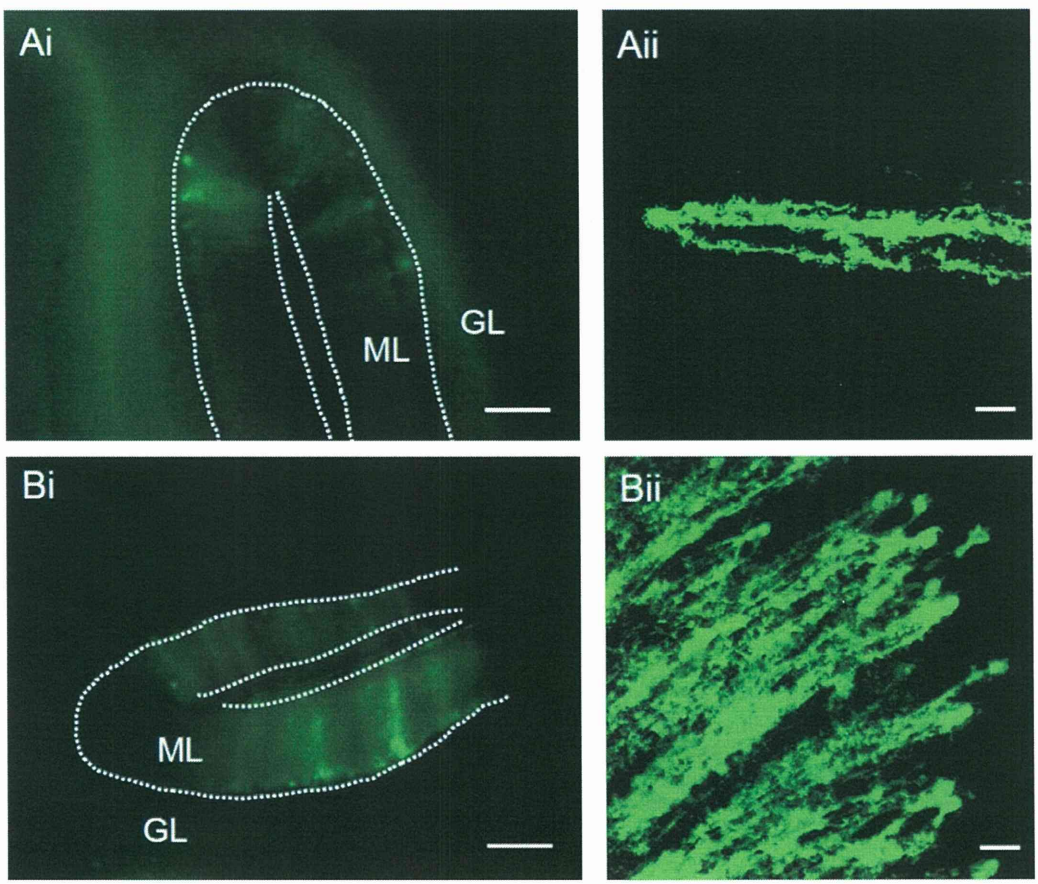

Fig. 4. Background infection. (Ai and ii) Cerebellar cells infected by ZZ Sindbis-Venus without antibody. Note that these cells were judged as Bergmann glial cells on the basis of their positions and shapes. (Bi and ii) Cerebellar cells infected by the control DH-BB-Venus. Ai and Bi are the microscopic images of Venus fluorescence (scale bars, 100 μm). Aii and Bii are images enhanced with anti-GFP immunohistochemistry (scale bars, 20 μm). ML, molecular layer; GL, granular layer.

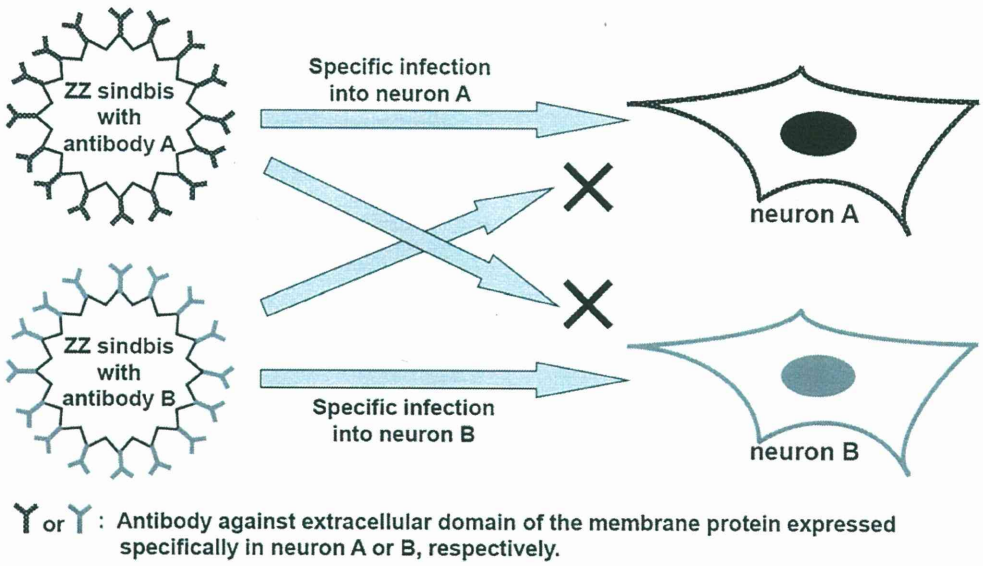

Fig. 5. Targeted infection of a neuron with the combination of ZZ Sindbis and cell-type specific antibodies (hypothesis).

idea that ZZ Sindbis-mCherry infects cells through the binding of an anti-GFP antibody to pHluorin.

ZZ Sindbis was generated by a recombination of the Sindbis virus, an enveloped virus with a single-stranded RNA genome (Ohno et al., 1997). The Sindbis virus vector has advantages in terms of its high expression levels and its rapid induction of foreign genes (Bredenbeek et al., 1993; Huang, 1996). Indeed, the gene expression induced by ZZ Sindbis could be observed within 12 h both *in vitro* and *in vivo*. Sindbis virus is also known to exhibit severe toxicity (Frolov and Schlesinger, 1994; Nargi-Aizenman and Griffin, 2001). However, the membrane properties, such as resting potential, input resistance and membrane time constant, of Sindbis-infected cells were the same as those of non-infected cells 12–24 h after inoculation (Ishizuka et al., 2006). Furthermore, Maletic-Savatic and colleagues reported that neurons infected with the Sindbis virus were physiologically healthy until at least day 3 postinfection (Maletic-Savatic et al., 1999). In our experiments, the 48-h post-transfection neurons with ZZ Sindbis showed no abnormality in their appearances (Figs. 2–4).

The Sindbis virus contains two envelope glycoproteins: E1, which mediates the fusion of the viral envelope with the target cell membrane, and E2, which mediates its binding to target cells (Garoff et al., 1994). As the ZZ domain was inserted into the E2 region, ZZ Sindbis showed reduced background infection levels (Ohno et al., 1997). Morizono and colleagues performed alanine substitutions for a number of amino acids that were reported to affect binding (Morizono et al., 2005). These mutations of E2 in ZZ Sindbis appreciably reduced the nonspecific infections. We used this modified envelope glycoprotein and found that ZZ Sindbis lacking antibodies rarely infected PC12 cells and hippocampus neurons (Figs. 1 and 2C). Unexpectedly, ZZ Sindbis was found to infect Bergmann glial cells in the cerebellum in an antibody-independent manner (Fig. 4A). This phenomenon may be attributable to the intrinsically robust tropism of the control Sindbis virus, DH-BB (Fig. 4B). The insertion of the ZZ domain into the E2 region (Ohno et al., 1997) and alanine substitutions of the amino acids that affect binding (Morizono et al., 2005) did not completely remove the background infection. This background infection was also maintained even in the case of injection with excess heparan sulfate (data not shown), which has been reported to play a key role in the binding of the Sindbis virus to target cells. It appears that Bergmann glial cells express some unidentified receptors that are involved in this background infection by the Sindbis virus. Further investigations are necessary to improve the specificity of the recombinant Sindbis virus displaying the IgG-binding domain.

In principle, the antibody-displaying viral vector particles can reach the target cells from their extracellular sides. Thus, we used antibodies that bind to the extracellular domains of the membrane proteins. For example, for the *in vitro* assay, we used an antibody that was directed against an extracellular region of the human $p75^{NTR}$, of which 15/16 amino acid residues are identical to those in rats. In the case of the *in vivo* assay, the anti-GFP antibody that was available for immunohistochemistry was used, and the targeted neurons expressed synaptopHluorin, in which a pH-sensitive derivative of GFP (pHluorin) is connected to the lumenal domain of a vesicular membrane protein, VAMP-2 (Miesenböck et al., 1998; Yuste et al., 2000). The pHluorins, which are recognized by the anti-GFP antibody, are expected to face the extracellular space, as a number of VAMP-2 molecules are also distributed in the plasma membrane (Takamori et al., 2006; Walch-Solimena et al., 1995).

The functions of neural cells have been characterized by membrane proteins including channels, transporters and receptors. Some adhesion molecules, such as members of the cadherin family, the integrin family, and the immunoglobulin superfamily, which are expressed in the membrane, are known to play important roles in axon guidance (Goodman, 1996; Maness and Schachner, 2007; Nakamoto et al., 2004; Yu and Bargmann, 2001) and synaptic connections (Takeichi, 2007; Rohrbough et al., 2000). Gene transfer techniques that utilize antibody-displaying viral vectors and the development of various antibodies that target the extracellular domains of membrane proteins could become powerful tools for neuroscience. Although the effectiveness of transfection could be improved by increasing the viral titer, current methods have advantages in labeling a small number of the type-identified cells with high specificity.

5. Conclusions

Here, we showed that ZZ Sindbis can deliver genes with high specificity both *in vitro* and *in vivo*. These data indicate the potential of antibody-displaying viral vectors for transferring a gene to a specific subset of neural cells in the CNS. This result was achieved by selecting an appropriate antibody, although more studies of this

type of viral vector are needed, especially regarding the relationship between the antibodies and targeted neural cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neures.2011.08.013.

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チャネルロドプシンを用いた視覚再生

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チャネルロドプシンを用いた視覚再生

富田　浩史・菅野江里子

〔要　約〕

走光性を示す緑藻類“クラミドモナス”は「チャネルロドプシン-2（ChR 2: channelrhodopsin-2）」と呼ばれる特徴的な機能を有する光感受性物質を持つ。この原始的な光感受性物質を利用することによって，従来光感受性を持たない神経細胞に光を受け取る能力を与えることができる。眼球と脳を連絡する神経節細胞は，失明後も網膜に残存しており，ChR 2 を用いてこれらの細胞に光を受け取る能力を与え，網膜神経節細胞を有効利用することによって，視覚機能を回復できる可能性がある。

はじめに

網膜色素変性症（RP: Retinitis Pigmentosa）の原因は，光受容に関与する視細胞あるいは網膜色素上皮細胞に特異的に存在するタンパク質をコードする遺伝子の変異である。RP による視細胞変性の機序は未だ不明な部分が多く，視細胞変性を阻止する治療法はない。RP の網膜組織像の特徴は，視細胞の消失であり，内顆粒層，神経節細胞層など他の細胞は，正常である（重症な RP では神経節細胞数が減少する）。

近年，視覚再建法として，残存する網膜細胞を電気的に刺激し，視機能を取り戻す人工網膜研究が盛んに行われている。アメリカ，ドイツ，日本で臨床試験が行われ，残存する網膜細胞を電気的に刺激することによって視覚が得られることが明らかとなっている。人工網膜研究から，残存する網膜細胞に何らかの方法で光情報を伝えることができれば，視機能を再建することができると考えられる。

緑藻類クラミドモナスより見出された，原始的な光感受性物質 ChR 2 タンパク質は，視細胞のロドプシンと異なり，ChR 2 タンパク質のみで光応答を作り出すことができる。本稿では，ChR 2 と視細胞変性後も網膜に残存する神経節細胞を利用した視覚再生法の概略を述べる。

Ⅰ．チャネルロドプシン-2 とロドプシン

池や田んぼ等に生息する緑藻類クラミドモナスは，2 本の鞭毛を有し，光のある場所に移動し光合成によりエネルギーを作り出し生活している。クラミドモナスは眼点と呼ばれる器官を有し，その眼点で光受容に重要な役割を担っているタンパク質がチャネルロドプシン-2（ChR 2）である。ChR 2 タンパク質は，古細菌型ロドプシンファミリー（微生物が持つ光受容タンパク質の総称）に属し，ヒトロドプシンと同様に 7 回膜貫通型の膜タンパク質である。発色団としてビタミン A アルデヒド「レチナール」分子を持つことから，古細菌型「ロドプシン」と呼ばれているが，両者のアミノ酸にホモロジー（アミノ酸配列の類似性）はない。また，光受容様式についても大きな違いが見られる。ロドプシンでは，ロドプシンによる光受容の後，複雑な細胞内の化学

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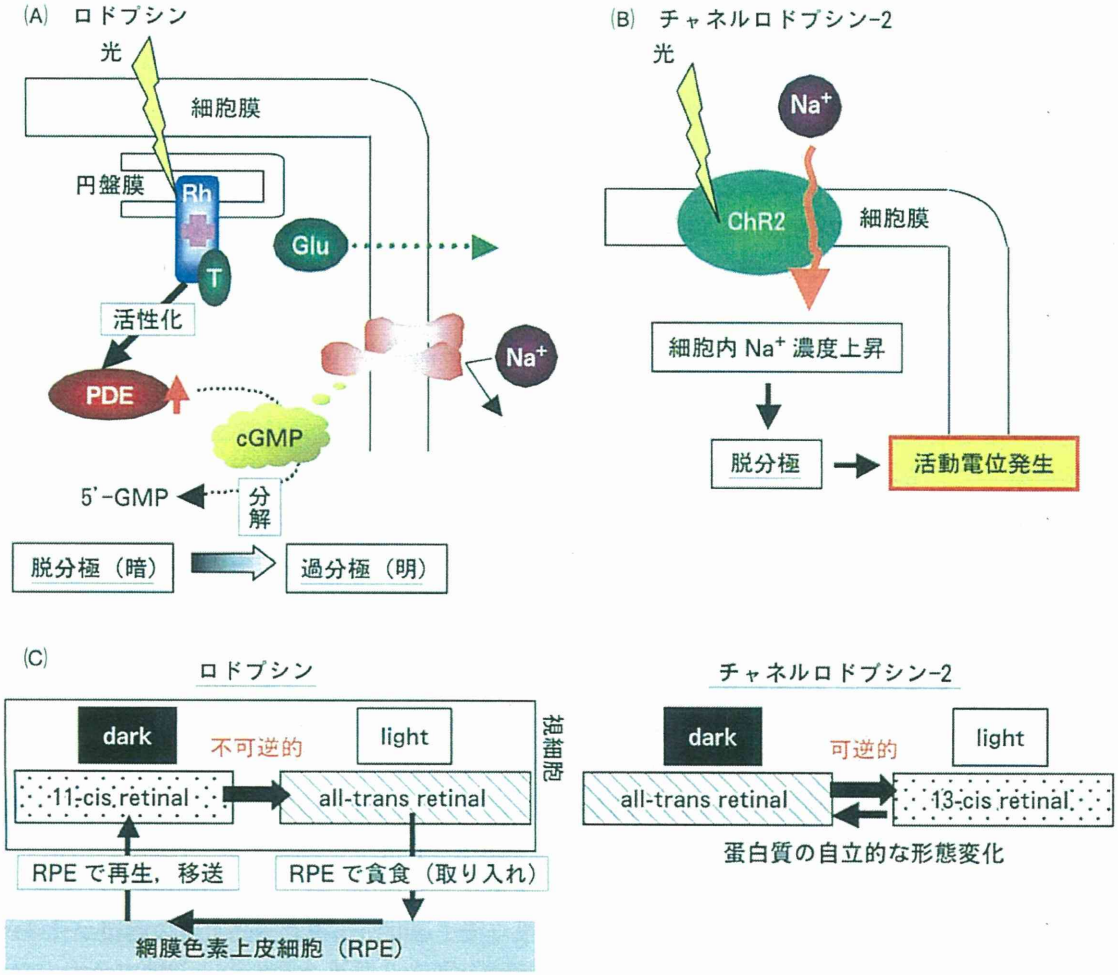

図1　ロドプシンとチャネルロドプシン-2（ChR 2）の比較

(A)　ロドプシンの光受容から過分極に至るまでの化学反応機構。レチナールの異性化によって活性化されたロドプシン（Rh）は，トランスデューシン（T）を活性化する。トランスデューシンはホスホジエステラーゼ（PDE）を活性化し cGMP を分解する。cGMP の分解に伴い，環状ヌクレオチド感受性陽イオンチャネル* は閉じ，ナトリウムイオンの流入が途絶える。その結果，視細胞は過分極する。

* 環状ヌクレオチド感受性陽イオンチャネル（cyclic nucleotide-gated cation channel）：環状ヌクレオチド（cAMP, cGMP）が結合しているときチャネルを開き陽イオンを流入させる。

(B)　ChR 2 の活動電位発生機構。光受容に伴い，ChR 2 の構造が変化し，ナトリウムイオンの透過性が亢進する。ナトリウムイオンの上昇に伴い，神経細胞は脱分極し活動電位を発生する。

(C)　レチナールの異性化と再生。ロドプシンでは，一旦，異性化した安定な all-trans 型レチナールは，視細胞および網膜色素上皮細胞に存在する酵素により，11-cis 型レチナールへと変換され再利用される。一方，ChR 2 では，光受容に all-trans 型レチナールを利用し，13-cis 型レチナールに異性化する。ロドプシンの 11-cis 型→all-trans 型が不可逆反応であるのに対し，ChR 2 では，all-trans 型から不安定な 13-cis 型への異性化であるために，暗状態になると all-trans 型に戻る，可逆性反応である。

変化を経て光受容が進行するのに対し（図1A），ChR 2 では，このような細胞内の連鎖反応を必要としない。視細胞のロドプシンの発色団（ビタミン A アルデヒド）の再生は，視細胞および網膜色素上皮細胞を経て再利用されるのに対し（不可逆反応），ChR 2 の発色団の再生は可逆反応である（図1B）。

このように，ChR 2 は単一のタンパク質の機能で，光受容に伴い細胞の興奮を引き起こすことが可能で，ChR 2 を導入することによって，どのような神経細胞にも光を受け取る能力を与えることができる[1]。

Ⅱ．遺伝子治療

1．チャネルロドプシン-2遺伝子導入による視覚再生法

視細胞の光受容は，ロドプシンだけでなく，種々の視細胞特異的なタンパク質の連鎖反応が必要で，視細胞以外の神経細胞にロドプシン遺伝子を導入したとしても光感受性を賦与することはできない。2002年Zemelmanら[2]は，神経細胞にアレスチン，ロドプシン，Gタンパク質αサブユニットの3種の遺伝子を導入することによって光受容能を賦与できることを報告している。しかし，視覚再生のための治療法として考えた場合，3種の遺伝子を単一の細胞に導入することは難しく，また，この方法は光感受性が低く実用化に至っていない。

一方，ChR 2は，光受容に伴い，細胞内に陽イオンを透過させる。すなわち，ChR 2を網膜神経節細胞に発現させた場合，光によって神経節細胞を脱分極させることが可能となる。正常な網膜において，視細胞で捉えられた光情報は双極細胞を経て，最終的に神経節細胞に伝えられ，神経節細胞が活動電位を発生させる。活動電位は軸索を伝播し，光情報として脳に伝えられる。網膜神経細胞の中で，脱分極によって活動電位を発生する神経細胞は，アマクリン細胞，神経節細胞があり，神経節細胞へのChR 2の遺伝子導入によって，神経節細胞は，光照射によって脱分極し活動電位を発生するようになる。このように，1つのChR 2遺伝子を神経節細胞に導入するのみで，神経節細胞自身が光に応答し，光情報を脳に伝えることが可能になる。また，Lagaliら[3]は，神経節細胞ではなく，ON型双極細胞にChR 2遺伝子を導入することによって，同様に視機能を回復させることに成功している。いずれにしても，ChR 2を神経細胞に発現させることによって，光で神経細胞を脱分極させることが「鍵」となっている。

2．ウイルスベクターの利用

ChR 2タンパク質を網膜で恒常的に発現させるためには，ChR 2遺伝子を網膜細胞内に送り込む必要がある。遺伝子を対象とする細胞に送り込む方法として，ウイルスベクターを用いる方法がある。アデノ随伴ウイルス，アデノウイルス，レトロウイルス，レンチウイルスなど様々なウイルスが遺伝子治療用のベクターとして開発されており，それぞれが特徴的な機能を持つ。なかでも，アデノ随伴ウイルスベクターは，神経細胞，筋細胞，肝細胞等の非分裂細胞に高効率で導入でき，しかも一度の投与で長期間（年単位）の遺伝子発現が可能である。レンチウイルスも同様の機能を有するが，アデノ随伴ウイルスが非病原性由来である点，そして，アデノ随伴ウイルスベクターで導入した遺伝子の多くがエピソーム*として存在する点から安全性が高い。

アデノ随伴ウイルスには1～11型の血清型があるが，ChR 2の神経節細胞への遺伝子導入には2型を用いている。2型は神経細胞に特異性が高く，網膜細胞では神経節細胞に高い親和性を持つためである。また，2型は広く遺伝子治療臨床研究（血友病やパーキンソン病など）に使用されているベクターであり，眼科分野においてもレーバー先天盲患者に対するRPE 65遺伝子を利用した遺伝子治療[4]が行われるなど，これまでにベクター自身の副作用は報告されておらず安全性の高い遺伝子治療ベクターと考えられている。

3．チャネルロドプシン-2によって得られる視覚

ChR 2遺伝子を含むアデノ随伴ウイルス2型ベクター溶液を遺伝盲ラットの硝子体内に投与すると，投与後1週間から視覚誘発電位の回復が見られ，視覚誘発電位の振幅は投与後8週間で最大となる[5]。回復した視覚誘発電位の潜時は，正常な網膜の潜時より短くなる。これは，正常な網膜では，光情報は「視細胞→内顆粒層（双極細胞）→神経節細胞→脳」と伝えられるのに対し，ChR 2を神経節細胞に導入した網膜では，「神経節細胞→脳」というように，「視細胞－内顆粒層」を経由しないためである（**図2A, B**）。光応答特性は，正常なラットと同等であることや，行動学的解析から，得られる視覚機能は低い空間周波数（視覚1度当たりの正弦波数）領域で高いコントラスト感度を持つことが示されている[6),7)]（**図2C**）。しかしながら，ChR 2タンパク質の特性として，感受波長ピークは460 nm付近（青色）で，緑，赤色などには反応しないこと（**図3A**），また，光感受性が正常な網膜と比べ極めて低いこと（**図3B**）などが問題点として挙げられている[7]。

4．遺伝子治療の安全性

ChR 2は元来ヒトが持たないタンパク質であり，恒常的に網膜で発現させることによって免疫応答を惹起する可能性が考えられる。動物実験で使用され

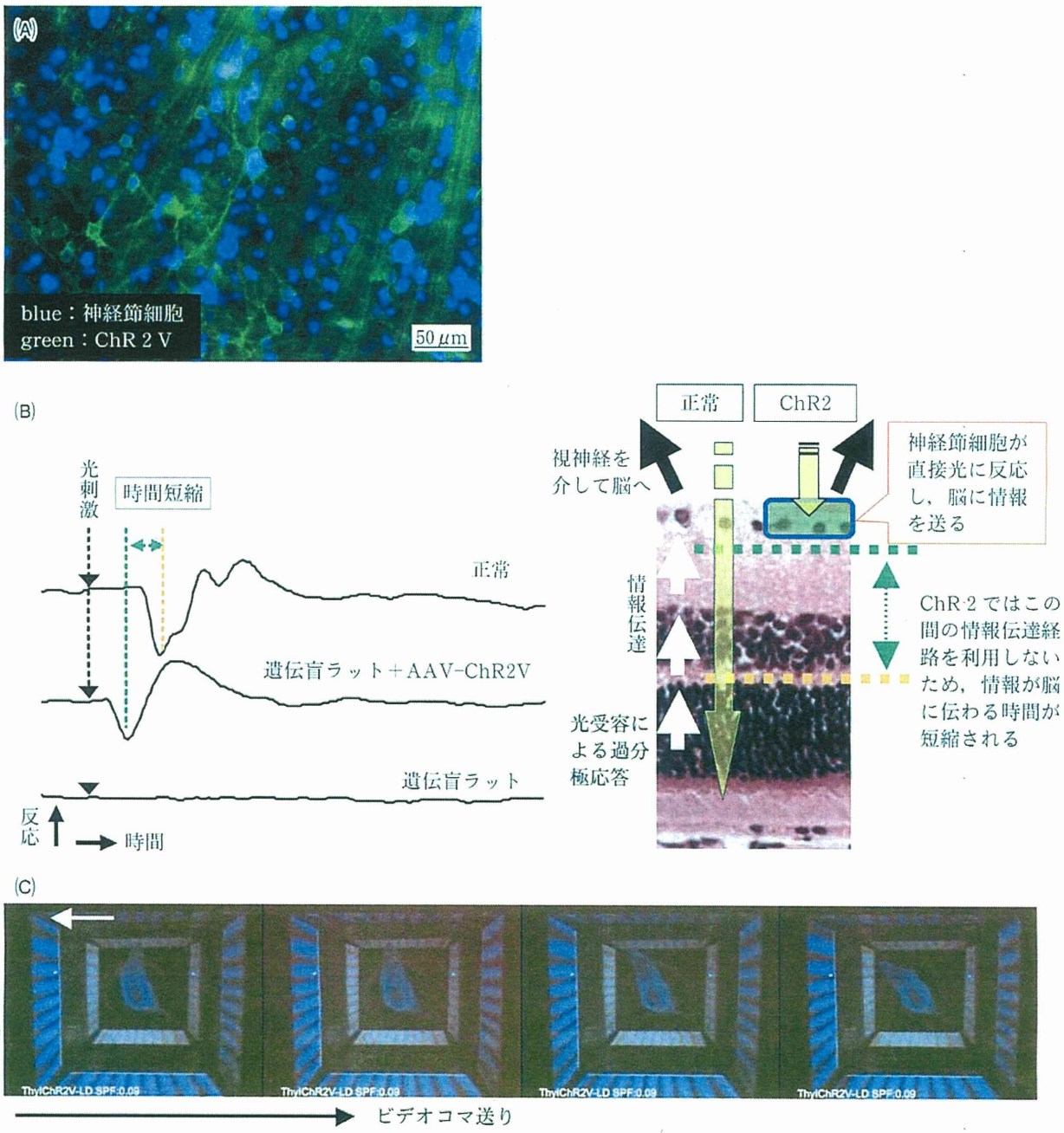

図2　ChR 2遺伝子導入ラット

(A)　網膜伸展標本。上丘に蛍光色素（青色）を注入し，網膜神経節細胞を逆行性標識した。ChR 2遺伝子に蛍光タンパク質（Venus）を融合しているため，ChR 2の発現は緑色の蛍光タンパク質として可視化される。
(B)　遺伝子導入後の視覚誘発電位波形。ChR 2を神経節細胞に発現するラットでは，潜時が短縮される（緑色矢印）。
(C)　回転する縞模様を用いた行動学的評価。ChR 2を発現する遺伝子改変ラットは，視細胞が存在しないにも関わらず，縞模様の回転を追うことができる（写真はビデオのコマ送り画像。矢印は縞模様の回転方向）。

ているウイルスベクターは，強力にChR 2の発現を誘導するように，転写活性の強力なプロモーター*が使用されている。また，このプロモーター*は細胞特異性を持たないために，万が一，標的細胞（神経節細胞）以外にウイルスが感染した場合，予期しない臓器でChR 2が発現する可能性がある。

ラットを用いた安全性研究では，ウイルスベクターを硝子体内に投与後6ヶ月で，一部のラットで小腸，肺にChR 2の発現が確認されている。これは眼内という閉鎖された空間であっても，また，自己複製能のないウイルスベクターであっても，全身への拡散の可能性がゼロではないことを示している。他臓