NOVEL CNS GENE DELIVERY SYSTEMS

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Dedicated with love to my husband and my parents

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SUMMARY

Gene therapy for neurological disorders requires carriers for the therapeutic genes which can safely and efficiently carry the genes into desired cell types. To date, a large number of viral and non-viral gene transfer vectors are used in the CNS gene delivery, and significant advancements have been achieved, which bring closer to reality the efficacious amelioration, even cure of CNS diseases.

However, at the present stage, none of the vectors can satisfy all the requirements of an ideal CNS gene delivery vector. The aim of this study was to exploit suitable gene carrier which has the potential for CNS gene delivery and to improve their performances in terms of efficiency, biosafety, and specificity. Both non-viral and viral vectors were involved in this research.

For the non-viral vector part, a newly developed biodegradable polymer, PPE-EA, was adopted in CSF gene delivery. The gene transfer efficiency, distribution, cytotoxicity and tissue response of this polymer were studied to evaluate the bioavailability of it in CNS gene therapy. The results established the potential of PPE-EA as a biocompatible gene carrier to achieve sustained gene expression in CNS.

For the viral vector part, a new baculovirus vector, BV-CMV E/PDGF, was constructed by utilizing a hybrid neuronal specific promoter, CMV E/PDGF, to drive the model gene expression. This recombinant baculovirus vector offered neuronal specific gene expression in primary neural cells and in rat brain. On the other hand, the transport profile of this recombinant baculovirus was systemically studies in several CNS pathways for the first time. Bidirectional axonal transport and transneuronal transport was detected in different CNS circuits.

In summary, the first part of this study established a DNA controlled release system in CSF, based on the new biodegradable polycation, PPE-EA. In the second part, a novel baculovirus vector accommodating a hybrid neuronal specific promoter successfully realized the neuron-targeted gene expression in the rat brain, while previously used

baculoviruse vectors bearing viral promoter were tested to be very poor in neuronal transfection. This modification would greatly widen the availability of the baculovirus as a CNS gene delivery vector. Finally, the delineation of the axonal transport paradigm of baculovirus contributed to our knowledge of its particular attributes in CNS, which is very important in terms of manipulating the transgene expression to fulfill the specific therapeutic requirement of a certain neurological disorder.

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LIST OF PUBLICATIONS

1. **Y Li**, J Wang, C Lee, CY Wang, SJ Gao, GP Tong, YX Ma, H Yu, H-Q Mao, KW Leong and S Wang, *CNS Gene Transfer Mediated by A Novel Controlled Release System Based on DNA Complexes of Degradable Polycation PPE-EA: A Comparison with Polyethylenimine/DNA Complexes.* Gene Therapy (2004), Vol.11, 109–114.

2. **Li Y**, Wang X, Guo H, Wang S. *Axonal transport of recombinant baculovirus vectors.* Mol Ther. 2004 Dec;10(6):1121-9.

3. **Li Y**, Yang Y, Wang S. *Neuronal gene transfer by baculovirus-derived vectors accommodating a neurone-specific promoter.* Exp Physiol. 2005 Jan;90(1):39-44.

4. L Shi, GP Tang, SJ Gao, YX Ma, BH Liu, **Y Li**, JM Zeng, YK Ng, KW Leong and S Wang, *Repeated Intrathecal Administration of Plasmid DNA Complexed with Polyethylene Glycol-Grafted Polyethylenimine Led to Prolonged Transgene Expression in the Spinal Cord*. Gene Therapy (2003), Vol. 10(14), 1179-1188.

5. Tang GP, Zeng JM, Gao SJ, Ma YX, Shi L, **Y Li**, Too H-Pb and Wang S, *polyethylene Glycol modified Polyethylenimine for Improved CNS Gene Transfer: Effects of PEGylation Extent*, Biomaterials (2003), Vol. 24(13), 2351-2362.

Patent in Process:

PCT/SG2004/000089, Method of Using Baculovirus Vectors for Gene Delivery to Neurons

ABBREVIATION

AAV	Adeno-associated virus
AcMNPV	Baculovirus Autographa californica multiple nuclear polyhedrosis virus
BV	Budded virus
β-Gal	β-galactosidase
CNS	Central Nervous System
CPs	Cationic polymers
DAF	Decay accelerating factor
GCL	Ganglion cell layer
GFAP	Glial fabrillary acidic proteins
HSV	Herpes simplex virus
INL	Inner nuclear layer
ITRs	Inverted terminal repeats
LGB	lateral geniculate nucleus
MNPV	Multicapsid nucleopolyhedroviruses
MOI	Multiplicity of infection
NeuN	Neuron-specific nuclear protein
NPVs	Nucleopolyhedroviruses
OBs	Occlusion bodies
ODVs	Occlusion derived virus
ON	Optic nerve
PEI	Poly(ethylenimine)
PLL	Poly(L-lysine)
PPE-EA	Poly(2-aminoethyl propylene phosphate)
CMV	Cytomegalovirus promoter
PDGF	Platelet derived growth factor
PVC	Primary visual cortex
RLU	Relative luciferase unit
SC	Superior colliculus
SN	Substantia nigra
SNPV	Single capsid nucleopolyhedroviruses

TH Tyrosine hydroxylase

CHARPTER 1. GENERAL INTRODUCTION

A large number of CNS diseases, such as neurodegenerative diseases, some of the brain tumors and traumatic brain are considered to be incurable to date. Gene transfer into the central nervous system (CNS) offers the prospect of manipulating gene expression for studying neuronal function and eventually for treating these incurable neurological disorders. There are two different approaches currently being utilized for the delivery of genetic materials in gene therapy: viral and non-viral gene delivery systems. Due to the unique attributes of the CNS, there are some obstacles to overcome in achieving efficient CNS gene delivery. One of the major obstacles is that CNS is more vulnerable and sensitive to the treatment imposed on it, which underscores the importance of developing safe gene delivery vectors and therapeutic administration methods. Another obstacle is the great diversity of cell types in the CNS, many of which has critical physiological functions and is highly sensitive to changes. This particular feature emphasizes the necessity of targeted gene delivery to a specific cell type. Great successes have been made in developing and exploring new gene delivery vectors in both nonviral and viral systems, but each has their own limitations. In the following parts, the latest progresses in both nonviral and viral vectors will be reviewed and their existing problems will be discussed.

1.1 Nonviral gene delivery in CNS

The success of gene therapy is largely dependent on the development of the gene delivery vector. Nonviral gene delivery vectors can be broadly categorized into three groups: naked DNA, cationic polymer, and lipid. This thesis mainly focuses on cationic polymers

(CPs) capable of condensing large gene fragments into small structures and masking negative DNA charges, which is necessary for transfecting most cell types. CPs-based gene delivery systems have been viewed as an alternative to viral gene vectors for their relatively low toxic effects and a lack of immune reactivity. Other potential advantages of polymer gene delivery systems include their capability in accommodating large DNA plasmids, simplicity in preparation, flexibility in use and cell-type specificity after chemical conjugation of a targeting ligand (Niidome and Huang, 2002). Due to these advantages, a number of cationic polymers have been studied and reported to be capable of mediating gene transfection, such as poly(ethylenimine) (PEI) (Boussif et al., 1995;Lambert et al., 1996), Poly(L-lysine) (PLL) (Wolfert et al. 1999), Chitosans (Koping-Hoggard et al. 2001), Dendrimers (Tang et al. 1996), etc. Among these commonly used polycations, PEI is considered to be the gold standard of non-viral gene delivery due to its high transfection efficiency both *in vivo* and *in vitro*. In particular, PEI polymers, especially those with molecular weight of 25 kD, may mediate DNA transfection in terminally differentiated post-mitotic neurons (Abdallah et al. 1996;Goula et al. 1998;Lambert et al., 1996;Shi et al. 2003;Wang et al. 2001), which is very beneficial for gene delivery to neurons into the CNS. Abdallah et al. reported that after direct brain injection, PEI/DNA complexes can provide transgene expression levels comparable to those obtained with the HIV-derived vector or adenoviral vectors (Abdallah et al., 1996). However, PEI has displayed toxicity and low biocompatibility to various types of cells, including neurons and other cells in the nervous system (Lambert et al., 1996;Shi et al., 2003). On the other hand, biodegradable polymers are known for their low toxicity, high biocompatibility. However low transfection efficiency is their

common apparent shortcoming. Recently, a newly developed biodegradable polymer, poly(2-aminoethyl propylene phosphate) [PPE-EA], shows very potent transfection efficiency, low cytotoxicity and high biocompatibility in skeletal muscle gene delivery (Wang et al., 2001;Wang et al., 2002), comparable to PEI and PLL. Although the characteristics of PPE-EA listed above indicate that it may be suitable for CNS gene delivery, the bioavailability of PPE-EA in CNS has not been studied.

1.2 Viral gene delivery in CNS

While non-viral vectors are considered to be promising alternative to viral vectors, viral gene delivery systems nevertheless still occupy a dominant position in the gene therapy field, owning to its high transfection efficiency unreachable by non-viral systems both *in vivo* and *in vitro*. There are a lot of virus vector being utilized in CNS gene delivery, including adenovirus, herpes simplex virus (HSV), adeno-associated virus (AAV), lentivirus, etc. But all of these vectors have inherent drawbacks which greatly limit their availability. Although a lot of efforts have been made in improving the performance of these virus vectors, none of them can fulfill every requirement of an ideal CNS gene delivery vector. Adenovirus and HSV vectors are highly immunogenic, and all individuals have pre-existing immunity to both these viruses. A single intracerebral injection of either adenovirus or HSV results in dose-dependent inflammatory reactions in the brain leading to demyelination (Lawrence et al., 1999; McMenamin et al., 1998). For lentivirus, the restricted host range, low titers, and pathogenic characteristics are all amongst its limitations as a CNS gene delivery system. AAV vectors are characterized by the inability to consistently induce immune responses, however its feasibility is restricted

by the small transgene holding capacity (Jooss and Chirmule 2003).

In recent years, promising viral vectors, based on insect baculovirus seems promising in overcoming those barriers which existed in the viral systems mentioned above. The baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is a newly emerged viral vector utilized in mammalian gene delivery system. It transfects a wide range of cell lines derived from different species efficiently, and also mediates significant transgene expression in different organs such as liver, spleen, kidney, brain, etc, in some animal models (Ghosh et al., 2002; Huser and Hofmann 2003). Besides this, baculovirus has other advantages such as replication deficiency in mammalian cell, large insert holding capacity, and simple production procedure, etc. Sarkis (Sarkis et al., 2000) described the transgene expression in several neural cell types after intra-striatum injection. Lehtolainen (Lehtolainen et al., 2002) reported that baculovirus can efficiently transfect the cuboid epithelium of the choroid plexus in ventricles after injection into corpus callosum. To fully explore the potential of baculovirus vectors for CNS gene delivery, it is necessary to carry out more studies to both widen and deepen our knowledge in the attributes of this vector.

Although baculovirus can mediate CNS gene delivery, it shows very poor neuro-tropism in the Sarkis (2000) and Lehtolainen's study（2002）. Since neurons are the major target of gene therapy for many kinds of neurological disorders, this drawback will inevitably limits the availability of baculovirus during CNS gene delivery. Hence, the problem of how to achieve neuronal specific gene expression for the baculovirus vector will

extensively limit the utility of this vector. On the other hand, we are not clear about the transport paradigm of the baculovirus in the nervous system, which is an important factor to consider in its further application as a CNS gene delivery vector.

1.3 Objectives of the research

The purpose of this study was to exploit novel non-viral and viral gene delivery vectors that can satisfy the requirements of an ideal CNS gene delivery vector, that is, with low cytotoxicity and high biocompatibility, high transfection efficiency, as well as specific transgene expression. Although it may be difficult to achieve all the attributes in one vector, it is possible to improve the performance of a vector by improving on the apparent drawbacks and retaining the advantages at the same time.

The aim of the first part of the research was to evaluate the bioavailability of PPE-EA in CNS gene delivery by intra-cisternal injection into cerebro-spinal fluid. PEI was used for comparison in gene expression efficiency, distribution, and biocompatibility studies in CNS. This detailed and systematic study may determine whether PPE-EA can be utilized as a safe and efficient gene delivery carrier in CNS in the future.

In the second part, the purpose was to develop a baculovirus vector with neuronal specific transfection. We constructed a recombinant baculovirus vector, namely, BV-CMV E/PDGF, with neuronal specificity by using a hybrid neuronal specific promoter, CMV E/PDGF, to control dreporter gene expression. To test the neuronal specificity of this recombinant baculovirus, both *in vitro* and *in vivo* studies were adopted with BV-

CMV (CMV full length promoter was used) as a control. For the *in vitro* test, mixed primary neural cells were used to measure virus gene delivery, and cell tropism was compared between BV-CMV E/PDGF and BV-CMV. For the *in vivo* test, both BV-CMV E/PDGF and BV-CMV was injected directly into the striatum. The *in vivo* cell tropism and the duration of the transgene expression were studied. The efforts for increasing the neuronal tropism of baculovirus may widen its availability as an efficient CNS gene delivery vector.

The third part focused on investigating the transport aspects of baculovirus in the CNS striatal pathway and visual pathway. Virus particles were injected into striatum and vitreous body separately and the transportation was tested in different brain areas which have connection with the injection site, at the DNA level by PCR and the protein level by luciferase activity assay. The clarification of the transport paradigm of baculovirus in CNS tissue may aid the design of targeting gene delivery to those brain regions that are not reachable by a traditional strategy of direct administration.

CHAPTER 2. LITERATURE REVIEW

2.1 CNS gene delivery systems

The development of a gene delivery system is one of the most important technological challenges for the goal of effective clinical therapy for CNS protection and repair. Many improvements in safety, efficacy, stability and regulatability of gene transfer to the brain are needed before realizing the purpose of clinical therapy. Advances in the modification of vectors for gene delivery offered selective and specific delivery modalities. But, given the complexity of the brain structure and diversity of CNS disorders, more efforts should be made to develop and explore new vectors. CNS has some unique attributes, including the post-mitotic nature of neurons, heterogeneity of cell types, critical functions of specific neuronal circuits, limited access, volumetric constraints, and presence of the blood-brain barrier, which are all challenges not usually at issue in gene therapy for other organs. (Costantini et al., 2000) Thus, the genes and tools for gene delivery should be tailored to meet the special therapeutic goals.

In this chapter, the nonviral and viral vectors commonly used in CNS gene delivery will be reviewed.

2.2 Nonviral gene delivery systems

Nonviral gene delivery systems can be categorized into three groups: 1) naked DNA delivery facilitated by physical methods, such as gene gun, electroporation, and ultrasound, etc; 2) gene transfer mediated by cationic polymers, such as PEI, PLL, chitosan, etc; 3) gene transfer mediated by lipids, such as N-[1-(2, 3-dioleoyloxy)propyl]-N',N',N'-trimethyl-ammonium chloride (DOTAP). In the brain, nonviral vectors induce

nearly no immune response or toxic effects. However, there is a low efficiency of expression of introduced genes compared with viral vectors (Brooks et al., 1998).

The following sections describe general profiles of PEI, naked DNA and PPE-EA, in terms of their use as gene delivery vector.

2.2.1 PEI mediated gene delivery

2.2.1.1 PEI chemistry

Among nonviral gene carriers in use, the polycationic polymer, polyethylenimine (PEI), has shown high transfection efficiency both in vitro and in vivo (Abdallah et al., 1996;Boussif et al., 1995;Goula et al., 1998). PEI comes in two forms: linear and branched. The branched form is produced by cationic polymerization from aziridine monomers (Fig. 2-1) via a chain-growth mechanism, with branch sites arising from specific interactions between two growing polymer molecules. The linear form of PEI also arises from cationic polymerization, but from a 2-substituted 2-oxazoline monomer (Fig. 2-1)instead. The product (for example linear poly(N-formalethylenimine)) is then hydrolyzed to yield linear PEI(Godbey et al. 1999). PEI has repeated basic unites with a backbone of two carbons followed by one nitrogen atom and contains primary, secondary, and, in the case of branched PEI, tertiary amino groups, each of which can be potentialy protonated.

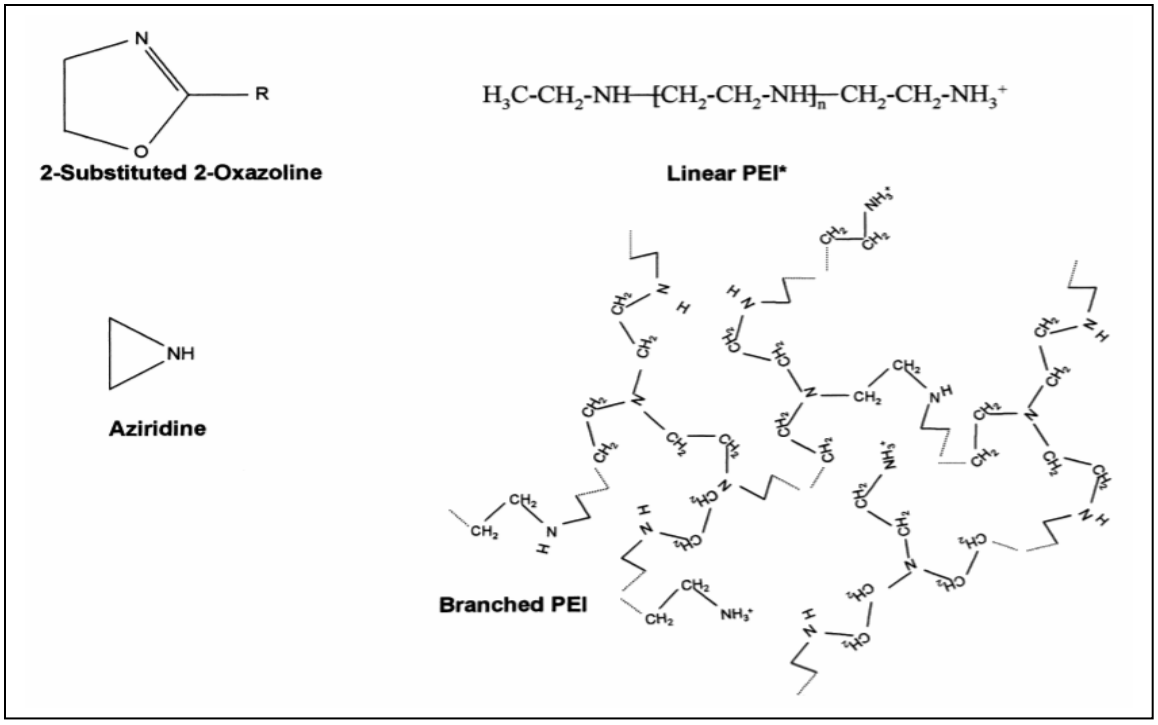


Fig. 2-1. Structures of PEI precursors and end products *Aziridine can also yield linear PEI under certain conditions. (adapted from Godbey, 1999)

2.2.1.2 PEI as an efficient gene delivery vector

The branched form of PEI has yielded significantly greater success in terms of cell transfection, and is therefore the standard form of PEI that is used for gene delivery. Highly branched polymers such as the 25-kDa PEI (Aldrich) and the 800-kDa PEI (Fluka) as well as polymers with lower degrees of branching (Fischer et al., 1999) are most frequently used. PEI mediates transfection by condensing DNA into nanoparticles/complexes, protecting DNA from enzymatic degradation, and facilitating the cell uptake and endolysosomal escape (Fig.2-2). PEI polymers are able to effectively complex even large DNA molecules (Campeau et al., 2001), leading to homogeneous spherical particles with a size of 100nm or less that are capable of transfecting cells efficiently in vitro as well as in vivo.

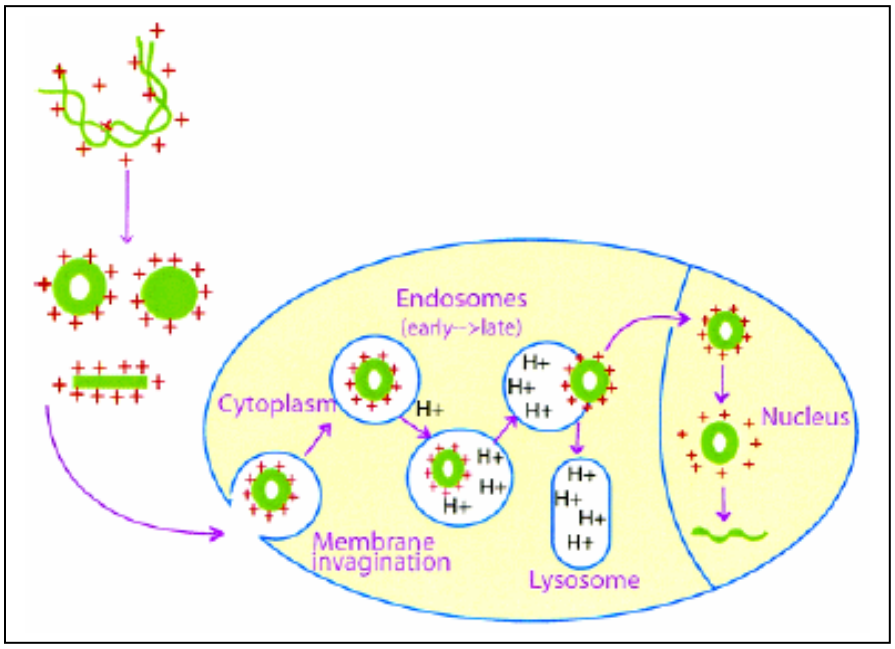


Fig. 2-2. Schematic representation of DNA uptake by mammalian cells. DNA is compacted in the presence of polycations into ordered structures such as toroids, rods, and spheroids. These particles interact with the anionic proteoglycans at the cell surface and are transported by endocytosis. The cationic agents accumulate in the acidic vesicles, increase the pH of the endosomes, and inhibit the degradation of DNA by lysosomal enzymes. They also sustain a proton influx, which destabilizes the endosome, and release DNA. The DNA then is translocated to the nucleus either through the nuclear pore or with the aid of nuclear localization signals, and decondenses after separation from the cationic delivery vehicle. (adapted from Vijayanathan, 2002)

PEI has been used as an efficient CNS gene delivery vector by several groups. The Feltz group successfully transfected primary central and peripheral neurons with antisense oligoneucleotides complexed by PEI (Lambert et al., 1996). High transfection efficiency was detected in mature mouse brain by using PEI/DNA complex with PEI molecular weight at 25-, 50- and 800- KD (Abdallah et al., 1996). But PEI 25- KD was tested to be the most efficient one, compared with other two. Following these reports, many works have been done using PEI for CNS gene delivery with PEI of different molecular weight,

by different injection pathway, or with different modifications (Goula et al., 1998;Shi et al., 2003;Tang et al., 2003).

2.2.1.3 Toxicity of PEI

The ratio of PEI nitrogens to DNA phosphates is important in terms of transfection efficiency and cell toxicity. Polymer/DNA complexes with an overall positive charge can activate complement, and reducing the +/– charge ratio of the complexes reduces complement activation as well as the amount of cell death associated with transfection (Plank et al., 1996). The huge amount of positive charges of PEI polymers results in a rather high toxicity, which is one of the major limiting factors especially for its use in CNS gene therapy (Shi et al., 2003).

2.2.2 Gene delivery by Naked DNA

2.2.2.1 Naked DNA mediated transfection

Significant amount of data indicates that the uptake and expression of naked DNA is a general property of animal cells within a tissue architecture. This phenomenon is common to cells of all three lineages: endoderm (eg, hepatocytes), mesoderm (eg, muscle), and ectoderm (eg, skin). Gene transfer to rodent brain by naked DNA has also been reported (Schwartz et al., 1996). In the brain, as in peripheral tissues, naked DNA vectors induce nearly no immune response or toxic effects. However, there is a low efficiency of expression of introduced genes compared with viral vectors (Schwartz et al., 1996). This property of transfection of neural cells is typically lost when the cells are removed and maintained in culture.

2.2.2.2 Physical methods of naked DNA delivery

The simplest way for administration of DNA is via direct injection of naked plasmid DNA into the tissue or systemic injection from a vessel. Use of naked DNA without any carrier molecule is also the safest method. Little attention needs to be paid on issues of complex formation and its safety assessment. So far, site of the direct injection includes skeletal muscle, liver, thyroid, heart muscle, urological organs, skin, brain and tumor (Nishikawa and Huang 2001). Systemic injection is also a convenient route for gene administration. However, owing to rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system, the expression level and the area after injection of naked DNA are generally limited. Various physical manipulations have been used to improve the efficiency. Electroporation, bioballistic (gene gun), ultrasound, hydrodynamics (high pressure) injection and other approaches have been tried (Li and Ma 2001).

2.2.3 PPE-EA gene mediated gene delivery (Wang et al., 2001;Wang et al., 2002)

2.2.3.1 PPE-EA Chemistry (Wang et al., 2001)

Biodegradable polycations are now emerging as a new generation of synthetic carriers. Biodegradable polymers, such as poly[α-(4-aminobutyl)-l-glycolic acid] (PAGA) have been reported to mediate successful gene transfection *in vitro* and *in vivo* (Maheshwari et al., 2000; Koh et al., 2000). These polymers show lower toxicity than PEI and PLL. Nevertheless, the average molecular weights of these reported polymers are relatively low, which limits the stability of the polymer-DNA complexes due to rapid hydrolysis.

PPE-EA is a new biodegradable polymeric carrier, which has a phosphate backbone and a β-aminoethoxy side chain (Fig. 2-3). PPE-EA was synthesized from a precursor polymer **1**, poly(4-methyl-2-oxo-2-chloro-1,3,2-dioxaphospholane). Reacting polymer **1** with 10% excess of benzyl *N*-(2-hydroxyethyl) carbamate in chloroform using 4-dimethylamino-pyridine (DMAP) as a catalyst yielded intermediate polymer **2**. PPE-EA was obtained as white powder (80%) after removal of the *N*-benzyloxycarbonyl group and followed by treating with chloric acid and precipitating in an access amount of acetone.

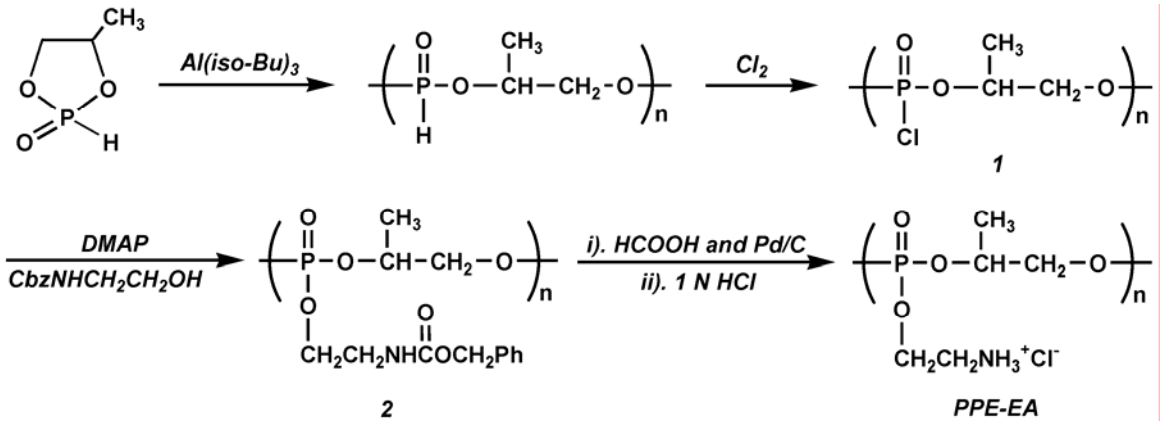


Fig. 2-3. Structures of PPE-EA precursors and end products. (adapted from Wang et al., 2001)

PPE-EA underwent degradation in PBS at 37 °C because of the hydrolytic cleavage of the phosphoester bonds in the backbone. The results of the study suggest a self-catalytic degradative mechanism involving nucleophilic attack of the phosphate bonds in the backbone by the pendant amino groups. After ten days of incubation in PBS, PPE-EA degrade to oligomers and fails to bind plasmid DNA. PPE-EA was designed with

nontoxic building blocks. The ultimate degradation products are expected to be α-propylene glycol, phosphate and ethanolamine, all with minimal toxicity profiles.

2.2.3.2 PPE-EA as an efficient gene delivery vector

Transfection mediated by PPE-EA was cell-type dependent. In HEK293 cells, PPE-EA yielded 45−105-fold higher gene expression than PLL-mediated transfection. But in COS7 and HeLa cells, it mediated about only 20- and 2-folds higher protein expression than PLL, respectively (Wang et al., 2001).

The *in vivo* gene transfer efficiency of the PPE-EA/DNA complexes was evaluated in mouse muscle using *LacZ* as a model gene. The β-galactosidase expression levels in mice received complexes with N/P ratios of 0.5 and 1, respectively, were compared with those of naked DNA injection. At a dose of 2 μg of DNA per muscle, PPE-EA/DNA complexes at N/P ratio of 1 mediated 13-fold and 6-fold higher gene expression than naked DNA expression at days 7 and 14, respectively. It is interesting to note that the complexes with a lower N/P ratio, 0.5 *versus* 1, were more effective, and a higher N/P ratios of 1.5 and 2 were ineffective (Wang et al., 2002).

2.3 Viral gene delivery systems

2.3.1 Properties of the ideal viral vector (Somia and Verma 2000)

The stumbling block of efficient gene therapy seems to be the vehicles that we used to deliver the therapeutic genes to the target tissues. The experiences in basic research and clinical phase I and II trials indicate that specific diseases and applications require their

specific viral vector system, depending on what is to be accomplished: long term correction of inherited genetic diseases or timely restricted and high level expression of a specific therapeutic gene product.

Ideal virus-based vectors for most gene-therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and toxicity. The adverse effects of gene therapy, such as the death of a 18-year-old boy resulted from severe inflammatory reaction caused by systemic delivery of adenovirus vectors in 1999, and the leukemia-like disease induced by gene therapy of human severe combined immunodeficiency (SCID)-XI disease using retrovirus vector in 2002, leading to intense concern about the safety issues of the viral vectors.

Tissue targeting, that is to deliver the transgene specifically into the tissue or organ of interest without wide spread vector dissemination, is highly desirable.

Easy production to commercial scale is also a big consideration for administration to humans. Given the great deal of cells that must be tranfected, a promise gene delivery vector should be easy to produce and purify at high titer.

The duration of transgene expression and vector immunogenicity are other important factors that influence the suitability of a vector for specific therapeutic applications. The expression of delivered genetic materials should be regulable in a precise way, for example, regulated expression in diabetes versus lifetime expression in hemophilia. The

potent immunogenicity and consequent short-lived transgene expression of early-generation adenovirus and HSV vectors are undesirable for many gene therapy applications. However, for cancer gene therapy, cellular toxicity and immunogenicity might enhance antitumour effects, and transient gene expression is advantageous in treatment of vascular and coronary artery disease.

Other attributes are large size capacity, faithful replication and segregation, infection of both dividing and non-dividing cells.

2.3.2 Characteristics of commonly used viral vectors

2.3.2.1 HSV-1 recombinant virus and amplicon vectors

Herpes simplex virus type 1 (HSV) is an enveloped virus bearing 152 kb of double-stranded DNA encoding over 80 genes, which has high infectivity for neurons and glia, as well as many other cell types. Two types of vectors are derived from HSV: recombinant virus vectors (RV) and amplicon vectors. HSV-RV vectors contain the full viral genome mutated in one or more virus genes to reduce toxicity and provide space fortransgenes (30–50 kb). Replication-conditional RV vectors can selectively replicate in and kill tumor cells in the brain. The HSV amplicon vector consists of a plasmid bearing the HSV origin of DNA replication, *oris*, and packaging signal, *pac*, which allows it to be packaged as a concatenate in HSV virions in the presence of HSV helper functions (Spaete and Frenkel 1982). These vectors can be packaged free of helper HSV virus by cotransfection with the HSV genome deleted for *pac* signals using a set of cosmids or BAC plasmid (Stavropoulos and Strathdee 1998;Saeki et al., 1998;Fraefel et al., 1996). In

neurons, HSV vectors are delivered by rapid retrograde transport along neurites to the cell body (Bearer et al., 1999;Sodeik et al., 1997), providing a means of targeting gene transfer to cells that are difficult to reach directly.

2.3.2.2 Adeno-associated virus (AAV) vectors

AAV consists of a non-pathogenic, small virion (20–24nm in diameter) containing a single-stranded DNA genome. AAV-based vectors have a 4.5 kb transgene capacity (Muzyczka 1992) and inverted terminal repeats (ITRs) that promote extrachromosomal replication and genomic integration of the transgene (Xiao et al., 1997). Integration of transgenes delivered by AAV vectors can be random or site-specific into human chromosome 19q13.3 (Kotin et al., 1990a;Balague et al., 1997;Walker et al., 1997;Weitzman et al., 1994;Yang et al., 1997). Long-term expression of transgene from AAV-based vectors is facilitated both by integration and maintenance as an episomal element within the host cell nucleus. AAV-based vectors produce high levels of transgene expression initially after injection into the CNS, predominantly in neurons (Bartlett et al., 1998;Lo et al., 1999b;Mandel et al., 1998;Kaplitt et al., 1994). Little toxicity has been observed with AAV vectors in brain and other tissues. Antibodies to AAV capsid proteins were low at 2 and 4 months after intracerebral injection and did not prevent transgene delivery upon re-administration of AAV (Lo et al., 1999a).

2.3.2.3 Adenovirus (Ad) vectors

The first generation of replication-defective Ad vectors constructed by deleting E1a, E1b and E3 genes, proved to have limited use in gene therapy, mainly due to a strong host

immune response to the viral antigens (Dai et al., 1995;Yang et al., 1994). Recently, high-capacity 'gutless' or 'mini-chromosome' Ad vectors have been generated that retain only the sequences necessary for packaging and replication of the viral genome, and lack all structural genes (Hardy et al., 1997b;Kochanek et al., 1996;Fisher et al., 1996). These gutless vectors have the advantages of increased transgene capacity (up to 37 kb) and propagation to high titers without contaminating helper Ad virus using a Cre–lox based recombinase system (Hardy et al., 1997a). *In vivo* studies have shown prolonged expression of transgenes delivered by these vectors with low host inflammatory response (Lieber et al., 1997;Kumar-Singh and Farber 1998;Morsy et al., 1998). Even in the presence of peripheral infection with adenovirus, there is virtually no immune response in the brain following direct injection of gutless vectors in rats. However, the high antigenicity of the Ad virion and toxicity of the virion penton protein81 remain as potential complicating factors with this vector system.

2.3.2.4 Retrovirus vectors

Retrovirus vectors are derived primarily from Moloney murine leukemia virus (MoMLV) (Mulligan 1993). These are enveloped RNA viruses which can transfer genes to a wide spectrum of dividing cell types.83 The vectors bear up to 8.5 kb of transgenes flanked by retroviral long terminal repeat (LTR) regions, a virion packaging signal (psi), and a primer binding site for reverse transcription. Retroviral RNA within the cell is reverse transcribed into double-stranded DNA and these sequences integrate randomly into the host cell genome. The use of retrovirus vectors for gene delivery to the nervous system has been limited by their ability to transfer genes only to dividing cells, yet have been

well suited for on-site delivery to neural precursors for lineage studies (Cepko et al., 1998) and to tumor cells for therapeutic intervention, and for *ex vivo* transplantation strategies.

2.3.2.5 Lentivirus vectors

The main advantage of lentivirus-based vectors is their ability to integrate into the host genome of nondividing cells, thereby providing the potential for a delivery system with stable expression even in post-mitotic neurons (Naldini et al., 1996c). The restricted host range, low titers, and pathogenic characteristics of HIV-1, itself, limit its utility as a gene delivery system for the CNS. In an effort to retain the positive attributes of HIV-1 and produce a safer and more versatile system, the HIV-1 vector is pseudotyped with the vesicular stomatitis virus G glycoprotein (VSVG), broadening the host range to include brain, liver and muscle cells (Naldini et al., 1996b;Zufferey et al., 1997;Naldini et al., 1996a;Kafri et al., 1997).

Table 1: The main groups of viral vectors

Vector	Genetic materials	Packaging capacity	Tropism	Advantages	Limitations
HSV-1	dsDNA	40kb	Strong for neurons	Large packaging capacity; Neuron tropism	Inflamatory response
AAV	ssDNA	<5kb	Broad	Non-inflammatory; Non-pathogenic	Small packaging capacity
Adenovirus	dsDNA	8kb	Broad	High transfection efficiency in most tissues	Potent inflammatory response
Retrovirus	RNA	8kb	Deviding cell	Persistant gene transfer in deviding cell	Only transduces deviding cells; in some applications, integration might induce oncogenesis
Lentivirus	RNA	8kb	Broad	Persistant gene transfer in most tissues	Integration might induce oncogenesis in some applications

2.3.3 Recombiant baculovirus vector

2.3.3.1 The baculovirus family

Baculoviruses (family Baculoviridae) constitute a group of double stranded DNA viruses that cause lethal diseases of arthropods (Miller 1997). Most baculoviruses have their hosts among lepidopteran insects, thus were used as biological pesticies (Mishra 1998). While most viruses are studied because they cause disease in humans or damage in food production systems, baculovirus studies were stimulated by the potential of these viruses to produce large amounts of recombinant proteins in insect cell culture and in insects (O'Reilly et al., 1992;Pennock et al., 1984;Smith et al., 1983). The best studied member of this family, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is a large enveloped virus with a double-stranded, circular DNA genome of ~130 kb. The complete sequence of the viral genome has been determined (Ayres et al., 1994). The most apparent characteristic of baculoviruses is the production of proteinaceous capsules, referred to as occlusion bodies (OB) or polyhedra. The occlusion bodies contain rod-shaped virions giving the name of the virus family Baculoviridae (*baculum* means rod in Latin) (Fig. 2-4). In Nucleopolyhedroviruses (NPVs) numerous virions are found in one occlusion body (polyhedron), which mainly consists of the polyhedrin protein. Within NPVs two morphotypes are recognized: single and multicapsid nucleopolyhedroviruses (SNPV and MNPV respectively). In MNPVs up to nine nucleocapsids are assembled in a single envelope, before a few tens of these are occluded into an occlusion body.

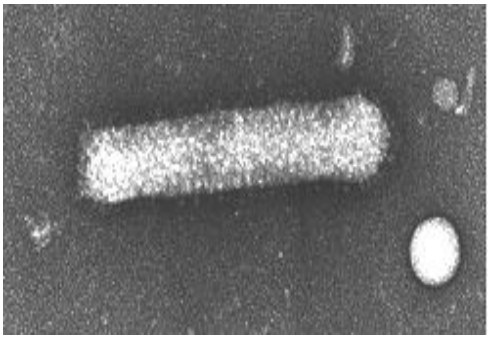


Fig. 2-4. EM picture of Baculovirus

2.3.3.2 Baculovirus infection cycle, replication and gene expression

Baculovirus infection starts when a susceptible insect larva ingests baculovirus occlusion bodies. The midgut lumen of lepidopteran larvae constitutes a highly alkaline environment in which OBs dissolve and the occlusion derived virions are released into the gut lumen. These virions pass through the peritrophic membrane and fuse with the microvillar membrane of the midgut epithelial cells where after they are transported to the nucleus, initiating the first replication cycle. Baculoviruses have a biphasic replication cycle, in which two genetically identical, but phenotypically distinct virus types are formed. The newly formed budded viruses (BVs) are initially released by budding through the plasma membrane of the infected cell. The insect tracheal system and the hemolymph play a major role in the transport of the BVs to other organs and tissues (Barrett et al., 1998b;Barrett et al., 1998a;Volkman 1997). Budded virions differ in several aspects from occlusion derived virus (ODVs), which are formed later in infection. (In cell culture BVs are 1000-fold more infectious than ODVs.) Budded virions are responsible for the systemic infection; ODVs facilitate viral spread from one individual insect to others. Budded virions enter the cell by endocytosis, followed by the fusion of the viral envelope and the endosome membrane. The fusion process is mediated

by a virus encoded essential glycoprotein, gp64, which is exclusively found in BVs (Blissard 1996). The ODVs are not released by budding, but acquire an envelope inside the nucleus, followed by occlusion in polyhedra. Finally, the infected cell ruptures and the lysis of both the nuclear and cellular membranes allow the release of the newly formed, mature polyhedra. The polyhedra are surrounded by an envelope composed of carbohydrates and specific proteins (Zuidema et al., 1989). The budded virions are the vector for producing recombinant proteins and are also used for mammalian gene delivery later (Fig. 2-5).

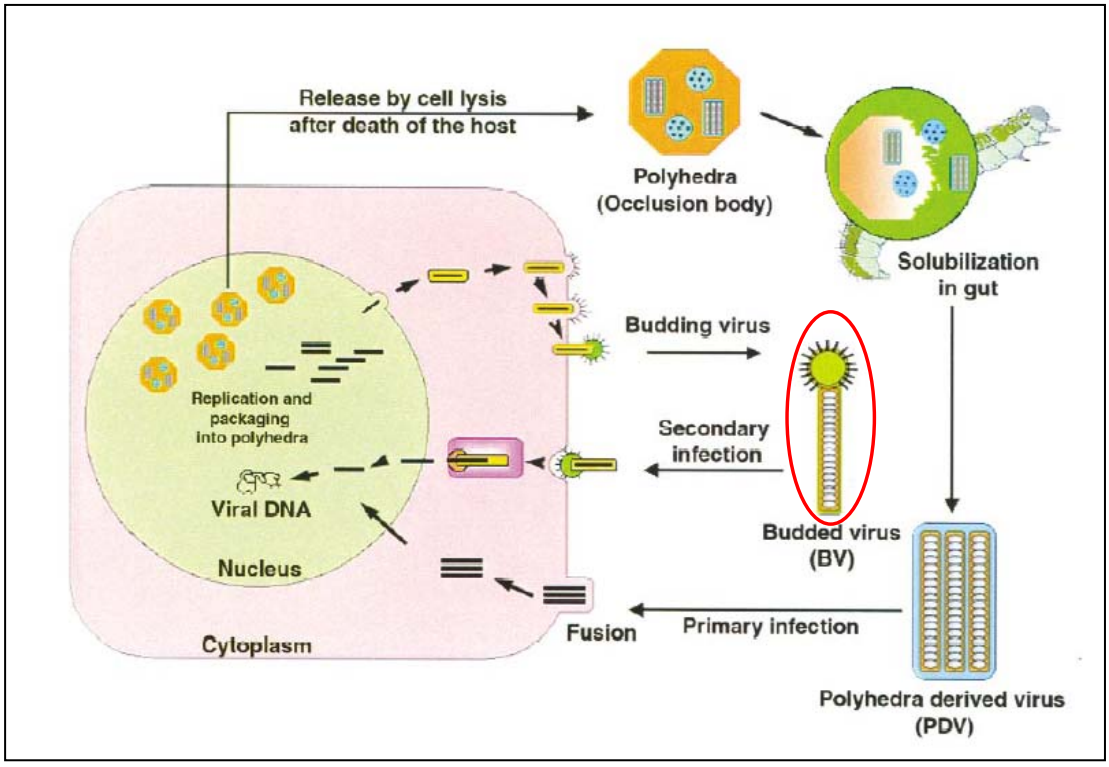


Fig. 2-5. Life cycle of Baculovirus (adapted from Ghosh, 2002)

2.3.3.3 Baculovirus-mediated gene transfer in mammalian cells

The application of recombinant baculoviruses for the expression of recombinant proteins in insect cells was first described in the early 1980s (O'Reilly et al., 1992;Pennock et al.,

1984;Smith et al., 1983). Since these initial reports, the baculovirus insect cell expression system has been extensively developed and used for the production of numerous recombinant proteins in insect cells. The most commonly used insect host cell lines include the Sf9 and Sf21AE lines originally derived from *Spodoptera frugiperda* pupal ovarian tissue (Vaughn et al., 1977) and theBTI-Tn-5B1-4 line, also known as 'High 5 cells', derived from *Trichoplusia ni* egg cell homogenates (Wang et al., 1994).

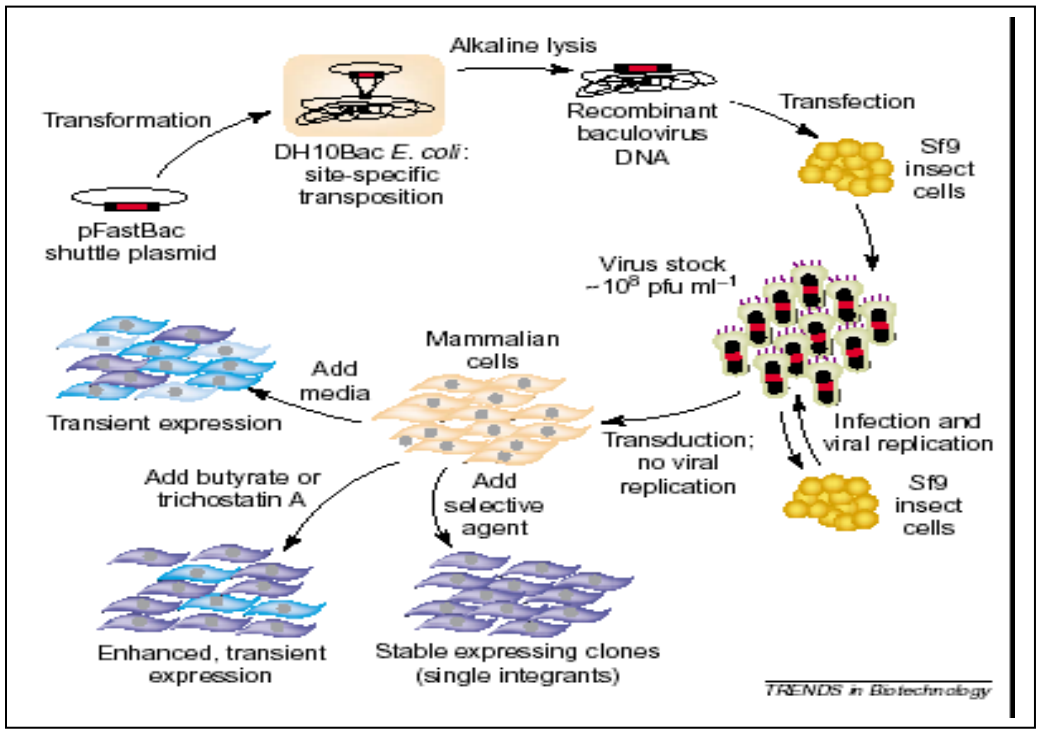


Fig. 2-6. Schematic diagram of baculovirus-mediated gene delivery (adapted from Kost, 2002)

The ability of baculovirus to enter certain mammalian cell lines was firstly reported by Volkman and Goldsmith in 1983 (Volkman and Goldsmith 1983), while no evidence of viral gene expression was observed. After that, two groups reported that recombinant viruses containing mammalian cell active promoter could be used to transducer mammalian cells in the mid 1990s. Hofmann et al. reported the successful transduction of

primary hepatocytes derived from different species, after incorporating a cytomegalovirus (CMV) promoter in the recombinant baculovirus (Hofmann et al., 1995). Boyce and Bucher also demonstrated transgene expression in several hepatocyte derived cell lines and primary cells by using a Rous sarcoma virus (RSV) promoter in the baculovirus vector (Boyce and Bucher 1996). Additional cell types were demonstrated to be successfully transduced in the following studies. Shoji et al.(Shoji et al., 1997) constructed a recombinant baculovirus bearing a hybrid promoter, consisting of a chicken β-actin gene enhancer element (CAG promoter), which efficiently transduced HepG2, HeLa and COS7 cells with the same level of transgene expression as that mediated by adenovirus at the same inoculation titres. Yap et al. established a hybrid baculovirus-T7 RNA polymerase system for transient transgene expression in several non-hepatocyte cell lines (Yap et al., 1997). This system is a two-component expression system, with one virus expressing T7 RNA polymerase and a second virus containing a reporter gene controlled by a T7 promoter. A broader spectrum of cell lines was also reported to be susceptible to baculovirus infection, with a CMV promoter. (Condreay et al., 1999)

Baculovirus mediated stable gene expression was also achieved by random or site-specific chromosomal integration of baculovirus genome into mammalian cell genome (Condreay et al., 1999;Merrihew et al., 2001;Palombo et al., 1998). Random integration was performed under antibiotic selection (Condreay et al., 1999;Merrihew et al., 2001), while site-specific integration was achieved by using a hybrid vector containing a transgene cassette composed of the β-galactosidase (β-Gal) reporter gene and the hygromycin resistance (Hygr) gene flanked by the AAV inverted terminal repeats (ITRs),

which are necessary for AAV replication and integration in the host genome (Palombo et al., 1998). With ITRs, the flanked sequences readily integrated into a defined region of the host cell genome located on chromosome 19q13.3 (Samulski et al., 1991; Kotin et al., 1990b). The list of transducable cell line is still expanding, as indicated by recent studies.

2.3.3.4 Baculovirus-mediated gene delivery in vivo

Baculovirus mediated gene expression in vivo were firstly performed in liver tissue.(Sandig et al., 1996) Although transgene expression level was very high in primary cultures, they failed to transduce liver from rat or mice by a variety of methods. This study elicits concerns of the inactivation of baculovirus by serum components. Follow up studies demonstrated that the complement system may be responsible for the inactivation. (Hofmann and Strauss 1998; Hofmann et al., 1999) To override this problem, researchers have explored several methods to avoid the stimulation and activation of the complement system. Complement resistant recombinant baculovirus were produced by fusing a decay accelerating factor (DAF) behind the gp64 envelope protein, and this virus vector successfully mediated the human factor IX expression in neonatal rat liver (Huser et al., 2001). Another successful strategy is to fuse a vesicular stomatitis virus G (VSV-G) protein downstream of the gp64 protein. This modified baculovirus vector displayed complement resistance, and hepatocytes and skeletal muscle were transduced by this vector after tail vein (Barsoum, Brown et al. 1997) and intramuscular injection (Pieroni, Maione et al. 2001) in mice. Successful viral transduction was also achieved in some tissues with low levels of complement such as the brain (Lehtolainen et al., 2002;Sarkis et al., 2000). These studies also showed that the viruses containing the CMV promoter

mediate expression primarily in non-neuronal cells, while with a RSV LTR promoter, transduced neurons can be detected.(Sarkis et al., 2000)

2.3.3.5 Advantages of Baculovirus as a gene delivery vector

2.3.3.5.1 Biosafety of baculovirus vectors

The investigation of interactions between baculoviruses and mammalian cells indicate that the former can be taken up but cannot replicated within mammalian cells.(Hartig et al., 1992;Volkman and Goldsmith 1983;Groner et al., 1984;Carbonell and Miller 1987;Carbonell et al., 1985;Doller et al., 1983;Tjia et al., 1983) Thus, no particular attention need to be paid to the risk of the replication-competent virus, which is a major problem in the use of adenovirus vectors. However, further studies will be required to determine whether any of the individual viral genes are expressed in mammalian cells.(Boyce and Bucher 1996)

2.3.3.5.2 Large insert capacity

The rod-shaped AcMNPV has a double-stranded, circular DNA about 130kb in size and the nucleocapsid structure can accommodate up to 100kb of foreign DNA. This advantage makes it possible to deliver multiple genes simultaneously.

2.3.3.5.3 Broad cell type specificity

More than 40 commonly used cell lines, including some primary cultures are reported to be successfully transduced (Ghosh et al., 2002;Kost and Condreay 2002). Moreover, it is noncytotoxic even at high multiplicity of infection (MOI).

2.3.3.5.4 Simple manipulating and producing procedure

The AcMNPV vectors are helper virus independent, thus, it is relatively simpler and easier to be constructed and produced at a large scale.(O'Reilly et al., 1992)

2.4 CNS circuits

2.4.1 Corticostriatal system

The cerebral cortex is supposed to provide the largest input to the basal ganglia by far, and nearly all regions of the neocortex project directly to the corpus striatum. The association areas in the frontal and parietal lobes have the heaviest projections compared with other cortical areas that innervate the striatum, such as the temporal, insular, and cingulate cortices. All of these projections, named collectively as the corticostriatal pathway, travel through the internal capsule to reach the caudate and putamen directly (Fig. 2-7).

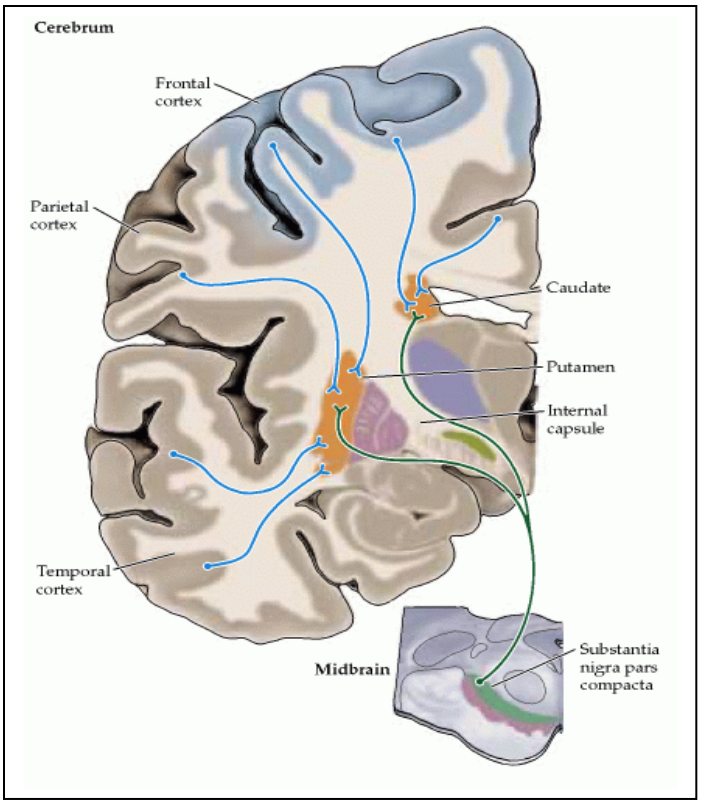


Fig. 2-7. Anatomical organization of the inputs to the basal ganglia. An idealized coronal section through the human brain, showing the projections from the cerebral cortex and the substantia nigra pars comparta to the caudate and putamen.(adapted from : www.utdallas.edu/~tres/ integ/mot3/motor_3.html)

Different functional area of the neocortex project to different parts of the striatum: the caudate and putamen. The caudate nucleus receives cortical projections primarily from multimodal association cortices, and from motor areas in the frontal lobe that control eye movements. The association cortices, as the name implies, do not process any one type of sensory information; rather, they receive inputs from a number of primary and secondary sensory cortices and associated thalamic nuclei. On the other hand, the putamen receives input from the primary and secondary somatic sensory cortices in the parietal lobe, the secondary (extrastriate) visual cortices in the occipital and temporal lobes, the premotor

and motor cortices in the frontal lobe, and the auditory association areas in the temporal lobe. The fact that different cortical areas project to different regions of the striatum implies that the corticostriatal pathway consists of multiple parallel pathways serving different functions.

2.4.2 Nigrostriatal system

The nigrostriatal pathway entails the dopaminergic cells in the pars compacta subdivision of substantia nigra and modulates the output of the corpus striatum. The medium spiny neurons of the corpus striatum project directly to substantia nigra pars compacta, which in turn sends widespread dopaminergic projections back to the spiny neurons. These dopaminergic influences on the spiny neurons are complex: The same nigral neurons can provide excitatory inputs mediated by D1 type dopaminergic receptors on the spiny cells that project to the internal globus pallidus (the direct pathway), and inhibitory inputs mediated by D2 type receptors on the spiny cells that project to the external globus pallidus (the indirect pathway). Since the actions of the direct and indirect pathways on the output of the basal ganglia are antagonistic, these different influences of the nigrostriatal axons produce the same effect, namely a decrease in the inhibitory outflow of the basal ganglia.

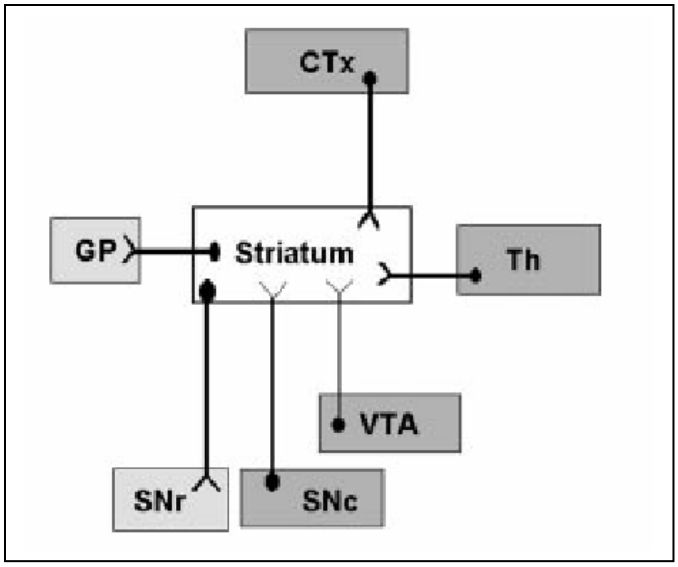


Fig. 2-8. Schematic diagram of major afferent and efferent projections from the striatum. Bold lines represent the strongest projections. CTx, cortex; GP, globus pallidus; SNc, substantia nigra pars compacta; Snr, substantia nigra pars reticulata; Th, thalamus; VTA, ventral tegmental area. (adapted from Costantini, 2000)

Parkinsonian symptoms may occur with the loss of the nigrostriatal dopaminergic neurons, resulting in an imbalance diminishing the disinhibition in the indirect circuit. SNc DA fibers would normally increase the total disinhibition of the thalamus through both the excitatory D1 receptors in the direct circuit and the inhibitory D2 receptors in the indirect circuit. Thus, with the lesions of the pallidum, as well as those of the SNc, the inhibitory outflow of the basal ganglia will be abnormally high, and thalamic activation of upper motor neurons in the motor cortex will be therefore less likely to occur, resulting in the appearance of Parkinson-like movement disorders.

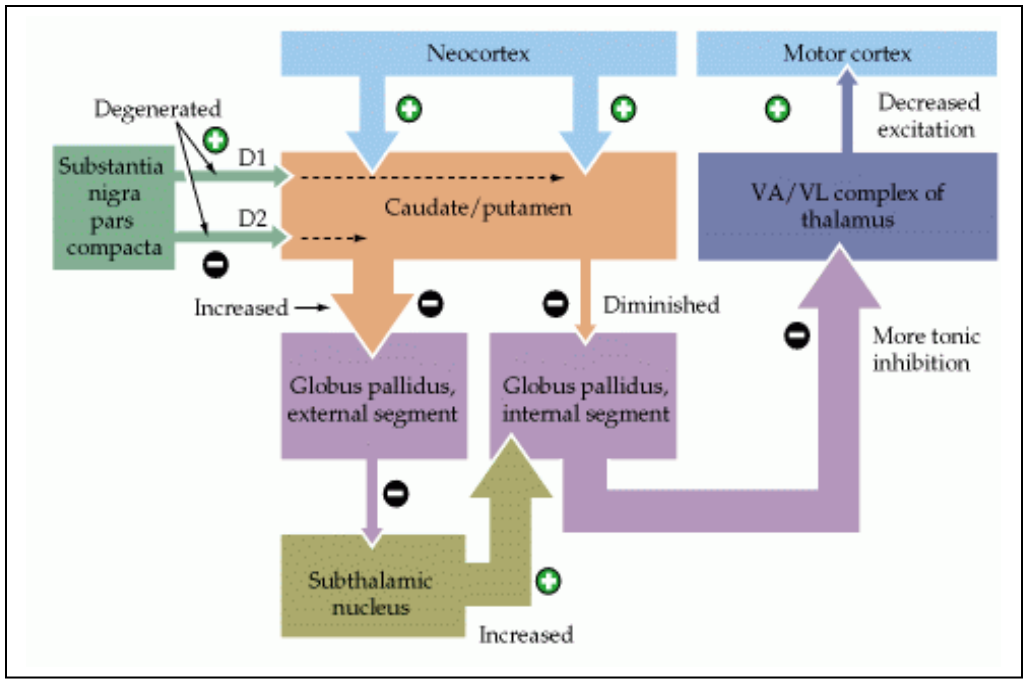


Fig. 2-8. An explanation for Parkinson's disease symptoms. The balance of inhibitory signals in the direct and indirect pathways is altered, leading to a diminished ability of the basal ganglia to control the thalamic output to the cortex. In Parkinson's disease, the inputs provided by the substantia nigra are diminished (thinner arrow), making it more difficult to generate the transient inhibition from the caudate and putamen. The result of this change in the direct pathway is to sustain the tonic inhibition from the globus pallidus (internal segment) to the thalamus, making thalamic excitation of the motor cortex less likely (thinner arrow from thalamus to cortex).

2.4.3 The visual system

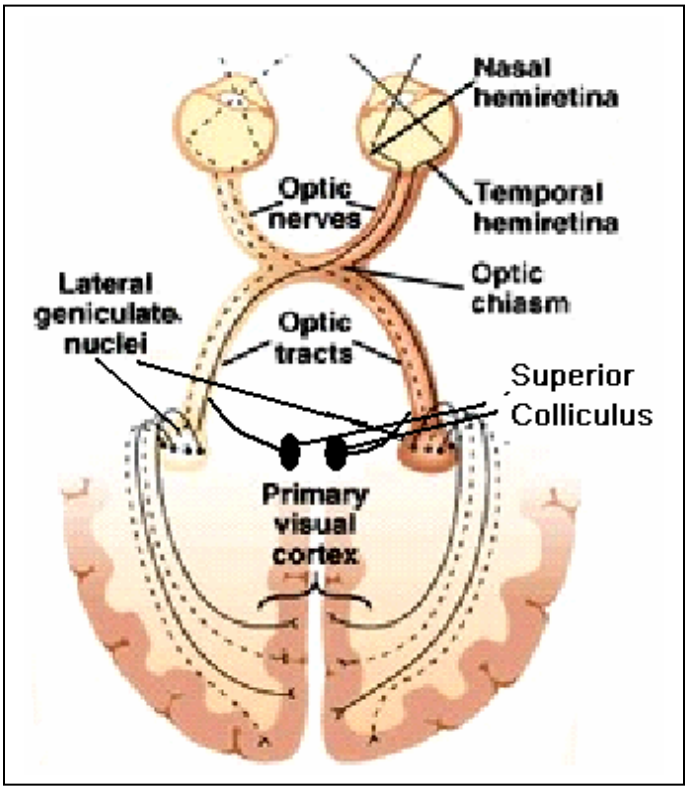


Fig. 2-9. Schematic picture of visual system

2.4.3.1 Retina

The retina is part of the peripheral components of the visual system; it is actually a displaced portion of the central nervous system (CNS) (Martin, 1991). It arises in the early embryonic development from a bilateral evagination of the prosencephalon, the primary optic vesicle. Each optic cup remains connected with the brain by a stalk, the future optic nerve (Fawcett, 1986). In the adult, the derivatives of the bilaminar secondary optic vesicle consist of an outer pigmented epithelial layer, the pigment epithelium, and an inner sheet, the neural retina or retina proper. The neural retina contains elements similar to those of the brain, and it may be considered to be a specially differentiated part of the brain (Fawcett, 1986; Yanoff and Fine, 1996).

In the retina (exclusive of the fovea, the papilla, and the ora serrata), ten parallel layers can be distinguished from outside inward: (1) pigment epithelium; (2) layer of rods and cones; (3) outer limiting membrane; (4) outer nuclear layer (ONL); (5) outer plexiform layer (OPL); (6) inner nuclear layer (INL); (7) inner plexiform layer (IPL); (8) ganglion cells layer (GCL); (9) optic nerve fibers layer (NFL); and (10) inner limiting membrane (Fawcett, 1986).

, Besides neurons, there are also some neuroglial cells, including Müller cells, astrocytes and microglia In the retina. The retinal neurons are supported and/or protected from injury by these neuroglial cells (Yanoff and Fine, 1996; Bron *et al.*, 1997).

2.4.3.2 Optic nerve

The axons of the ganglion cells leave the retina to become fibers of the optic nerve. The optic nerve leaves the eyeball at the optic disc and enters the cranial cavity through the optic canal. The two nerves meet each other at the optic chiasma where only fibers from the nasal halves of the two retinae cross. Fibers from temporal halves of the two retinae remain uncrossed. Distal to the chiasma, the 2 whole nerves are called the optic tracts. Each optic tract is composed of nerve fibers from the temporal half of the retina of the same side and the nasal half of the retina of the opposite eye (Fawcett, 1986). In their course across the retina, the afferent nerve fibers are not myelinated as this would obstruct light passing to the photoreceptors. Just after leaving the eye through the openings in the lamina cribrose, the nerve fibers acquire their myelin sheaths. The central artery and central vein reach the eyeball through the optic nerve (Fawcett, 1986).

2.4.3.3 Lateral geniculate nucleus (LGN)

Most of the nerve fibers of the optic tract terminate in the lateral geniculate nucleus (LGN), a large nucleus that forms a surface landmark on the ventral diencephalon. The LGN is one of the most prominent nuclei of the primary visual pathway, which provides a relay station for retinal axons synapsing with neurons of the geniculocalcarine pathway, transferring information from the optic tract to the optic radiation and thence to the visual cortex. Signals from the retina are transmitted to the cortex without much processing in the LGN (Martin, 1991; Bron *et al.*, 1997).

2.4.3.4 Superior colliculus

Besides projecting to the LGN, some optic tract axons also terminate in the superior colliculus (SC). The SC are small rounded elevations on the dorsal surface of the midbrain, separated by a vertical median groove. In lower vertebrates, the SC serves both vision forming and visual reflex function. In higher vertebrates, vision forming is one function of the visual cortex while the SC is more important in visuomotor function (Martin, 1991; Bron *et al.*, 1997).

2.4.3.5 Primary visual cortex

Nerve fibers which arise from the LGN relay through the geniculocalcarine tract (optic radiation) and project to the visual cortex for visual perception. The visual cortex is situated in both banks of the calcarine sulcus, at the medial surface of the occipital lobe. It is the place where objects in and out of their visual context are analyzed in detail.

Visual cortex is the first location in which signals from the two eyes converge onto a single cell (Daw, 1995).

CHAPTER 3. DEGRADABLE POLYCATION PPE-EA AS A NOVEL DNA CARRIER FOR CNS GENE TRANSFER: A COMPARISON WITH PEI

3.1 Abstract

Nonviral gene delivery systems based upon polycation/plasmid DNA complexes are quickly gaining recognition as an alternative to viral gene vectors for their potentials in avoiding problems inherent in viral systems. We investigated in this study the feasibility of using a recently developed biodegradable polycation, PPE-EA, as a controlled release system of plasmid DNA for gene transfer in the brain. PPE-EA/DNA complexes, as well as naked DNA and DNA complexed with polyethylenimine (PEI), a potent polycationic gene carrier that is not biodegradable, were delivered into the mouse cerebrospinal fluid by intracisternal injection. While transgene expression mediated by naked DNA was mainly detected in the brain stem, a region close to the injection site, higher levels of gene expression could be detected in the cerebral cortex, basal ganglia and diencephalons after using either PPE-EA or PEI. Transgene expression in the brain mediated by PPE-EA/DNA complexes persisted for at least 4 weeks, at a significant higher level than those offered by either naked plasmid DNA or polyethylenimine (PEI) 28 days after injection. To examine the biocompatibility of PPE-EA in the nervous system, in vitro cytotoxicity assay and in vivo histological examination were carried out. PPE-EA showed much lower toxicity to neural cell lines as compared to PEI and did not cause any pathological changes in CNS tissues. Thus, PPE-EA was verified to be a promising non-viral delivery vehicle for gene transfer in the CNS.

3.2 Introduction

Gene transfer into the central nervous system (CNS) offers the prospect of manipulating gene expression for studying neuronal function and eventually for treating neurological disorders. Many efforts have been made in CNS gene delivery, using either viral or nonviral vehicles. Polymer-based gene delivery systems have been viewed as an alternative to viral gene vectors for their advantages in inducing relatively low toxic effects and nearly no immune response. Other potential advantages of polymer gene delivery systems include capability of dealing with large DNA plasmids, simplicity in preparation, flexibility in use and cell-type specificity after chemical conjugation of a targeting ligand. Among these polymers, polyethylenimine (PEI) has shown high transfection efficiency both in vitro and in vivo (Lemkine and Demeneix 2001;Kircheis et al., 2001;Godbey et al., 1999a;Boussif et al., 1995). In particular, PEI polymers, especially those with molecular weight of 25 kD, may mediate DNA transfection in terminally differentiated non-dividing neurons (Abdallah et al., 1996;Godbey et al., 1999b;Goula et al., 1998;Lambert et al., 1996;Shi et al., 2003;Wang et al., 2001). After direct brain injection, PEI/DNA complexes can provide transgene expression levels comparable to those obtained with the HIV-derived vector or adenoviral vectors (Wang et al., 2001). However, the polymer has displayed some level of toxicity to various types of cells, including neurons and other cells in the nervous system (Abdallah et al., 1996;Godbey et al., 2001). This polymer and its DNA complexes may enter the cell nuclei (Wang et al., 2001); the accumulation of non-biodegradable, highly positively charged PEI in the cell nucleus is a concern because of its potential long-term harmful effects on cellular gene expression(Wang et al., 2002).

Recently, a new polymeric gene carrier has been developed (Nguyen et al., 2000). The carrier is based on a water soluble cationic polyphosphoester, poly(2-aminoethyl propylene phosphate) [PPE-EA]. The polymer degrades in PBS at 37 °C through the cleavage of the backbone phosphate bonds. The tissue response and cytotoxicity study demonstrated a better tissue compatibility of PPE-EA in mouse muscle compared to both PEI and poly-L-lysine, another commonly used polycationic gene carrier. The polymer readily forms complexes with plasmid DNA and the PPE-EA/DNA complexes formed act as a controlled release system for sustained release of plasmid DNA. PPE-EA functions in the system to increase DNA bioavailability by both protecting DNA from degradation and controlling the dispersion and retention of DNA in tissue, thus offering the possibility of enhanced gene transfer efficiency and prolonged gene expression. After intramuscular injection, PPE-EA/DNA complexes have resulted in enhanced gene expression compared to naked DNA injections (Ogris et al., 1999).

In considering the fact that even naked plasmid DNA could mediate gene transfer in the CNS, we have explored the feasibility of using PPE-EA/DNA complexes for gene expression in the brain after intracisternal injection in mice. We presented in this report that PPE-EA/DNA complexes functioned as a controlled release system in the brain, mediating prolonged gene transfer and was superior either naked DNA or PEI. We also showed further a lower cytotoxicity and better in vivo biocompatibility of PPE-EA compared with PEI.

3.3 Materials and Methods

3.3.1 Materials

Poly [2-aminoethyl propylene phosphates] (PPE-EA) was synthesized according to a previously reported method (Wang et al., 2002), and donated by Dr. Jun Wang form Johnhopkins Singapore. Polyethylenimine (branched PEI, average molecular weight of 25 kDa) was obtained from Sigma. Immuno-staining kit, Qiagen kit, Luciferase kit, and TUNEL kit were obtained from Dako, Qiagen, Promega, and Roche, respectively. (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma. Cell culture media were from Life Technologies.

3.3.2 Plasmid

The plasmid used was pCAGLuc (kindly donated by Yoshiharu Matsuura, National Institute of Infectious Diseases, Tokyo, Japan), encoding a firefly luciferase gene driven by a composite promoter CAG consisting of the CMV IE enhancer, chicken β-actin promoter and rabbit β-globin polyadenylation signal. All plasmid DNAs were amplified in *E.coli* (DH5 α)and purified according to the supplier's protocol (Qiagen, Hilden, Germany). The quantity and quality of the purified plasmid DNA was assessed by optical density at 260 and 280nm and by electrophoresis in 1% agarose gel. The purified plasmid DNA was re-suspended in TE buffer and kept at -80^0C in aliquots at a concentration of 1 mg/mL.

3.3.3 Preparation and characterization of DNA/polymer complexes

The PPE-EA/DNA complexes were prepared in 5% glucose solution. Plasmid DNA was diluted to a concentration of 0.2mg/ml. After vortexing, the appropriate amount of PPE-EA or PEI was added drop-wise into DNA solutions and the solutions re-vortexed. The

required amount of polymers, according to DNA concentration and number of equivalents needed, was calculated by taking into account that 1μg DNA contains 3nmol of phosphate, 1μL of 0.01M PPE-EA holds 10 nmole of amine nitrogen, and 1μL of 0.1M PEI holds 100 nmol of amine nitrogen. Polymer cation/DNA anion ratios were presented as the molar ratio of polymer nitrogen to DNA phosphate (N/P ratio).

For agarose gel electrophoresis, polymer DNA complexes mixed with a loading buffer were loaded onto an ethidium bromide containing 1% agarose gel. Gel electrophoresis was carried out at room temperature in TEB buffer at 80 V for 60 min. DNA bands were visualized by a UV (254 nm) illuminator.

For complex size measurements, a N4 Plus Submicron Particle Sizer (COULTER, USA) was used. Scattering light was detected at 90^{o} angle, running of 200 sec at room temperature. The refractive index medium (1.332) of 5% glucose was used for data analysis. The data obtained was analyzed in the Unimodal Analysis mode. This experiment was done under Dr. Tang Guping's guidance.

3.3.4 Atomic Force Microscopy (AFM)

Atomic Force microscopy was used for imaging the shape of the DNA and polymer/plasmid DNA complex at various charge ratios. The pRSVlus DNA was diluted with HEPES-Mg buffer (10mM $MgCl_2$, 40Mm HEPES, pH7.6 v/v=1/1) to a final concentration of 30 ng/ul. After incubation for 5-10 min at room temperature, two microliters of this solution was deposited onto the center of a freshly cleaved mica

surface. After being left standing for 10 min, the sample was rinsed with 2-5ml of deionized water. The sample was then blown dry with a gentle stream of nitrogen. PPE-EA/DNA complexes were prepared at nmol/nmol ratios of 0.5/1 and 2/1 and diluted with deionized water. After an incubation for 30 min at room temperature, three microliters of each aqueous complex solution of the 2 charge ratios (with final DNA amounts of 10 ng in each sample) were loaded onto the center of a fresh mica disk. After being left standing for 10 min, the samples were rinsed with 2-5ml of deionized water. The samples were then blown dry with a gentle stream of nitrogen. AFM (Multi Mode, Nanoscope III, Digital Instruments, Santa Barbara, CA) was used to image at room temperature in tapping mode, using a Si_3N_4 cantilever with a spring constant of 34N/m and a resonance frequency of about 280 kHz. Scanning was performed at a scan speed of 0.5-2 Hz. The resolution of 256×256 pixel images were captured *with scan size of 1-2 um*. The tip loading force was minimised to avoid structural changes to the samples. This experiment was done under Dr. Tang Guping's guidance.

3.3.5 Animals and injection procedures (Fig. 3-1)

Adult male Swiss mice (20-25g) were used throughout the study. Before each injection, animals were anesthetized by inhalation of ether. The intracisternal injection was done according to *Reijneveld* et al. A 10 μl Hamilton syringe connected to a 26-gauge needle was used, and a plastic stopper was set on the needle 4mm from the tip. The injection procedure was as according to *Reijneveld* et al. 10 μl of 5% glucose solution was given to each mouse, which contains 1μg of DNA condensed by the appropriate amount of PPE-EA. The animals were kept warm until recovered.

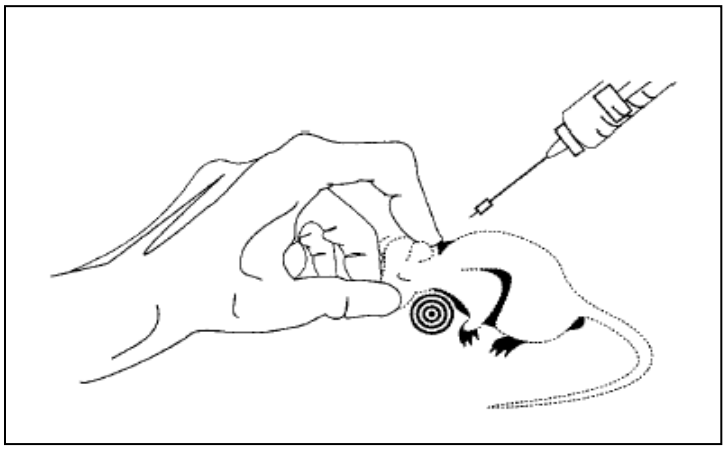


Fig. 3-1. Method of Intracisternal Injection

For all in vivo experiments, 6 mice were used for each group. Mice injected with PPE-EA/DNA complexes of different N/P ratios were sacrificed 3 days after injection. Animals used for time course experiments were sacrificed at different time points after intracisternal injection. Then brain tissues were harvested and used for luciferase activity assay.

3.3.6 Luciferase activity assay

Animals were anesthetized by inhalation of ether and then perfused transcardially with 0.1 M phosphate-buffered saline (PBS, 10 ml/mouse). After perfusion, the whole brain tissue were removed and separated into four parts: cortex, dosal ganglion/diencephalon, cerebellum and brain stem. The tissue samples were homogenized in 200 μl PBS by sonication (grade 6, 10 sec per sample) on ice. Cell lysates were centrifuged at 14,000 rpm at 4^0C for 10 min. 50 μl of supernatant was taken for the luciferase activity assay according to a written protocol included in the luciferase assay Kit (Promega).

Measurement was carried out in a single-well Luminometer (Berthold Lumat LB 9507, Germany) for 10 seconds.

3.3.7 Immune staining

For immumostaining, the mice were anaesthetized with ether-air mixture by inhalation and perfused intracardically with 10ml PBS followed by 10 ml per mouse of ice cold fixative solution (4% paraformaldehyde in PBS). The brain tissue sections were placed in 4% paraformaldehyde overnight at 4^{o}C, and then transferred to 20% sucrose in PBS over night at 4^{o}C.

A piece of sample was embedded on the cryostat sample stage with OCT medium, and then immersed in liquid nitrogen for 30 seconds. The sample was then transferred to the cryostat chamber for sectioning. After setting up on an ultra microtome at –20^{0}C (OT) and –15^{0}C (CT) respectively, sections at thickness of 25μm were cut. The sections were transferred to tail collagen coated slide and fixed with acetone. A goat anti-luciferase (1/200, in PBS 1% BSA, Dako) and FITC conjugated anti-goat IgG were used as the primary and secondary antibodies, respectively. After antibody incubation and washing, the sections were mounted in FluorSave (Calbiochem). Photographs were taken with a cooled CCD camera attached to the microscope.

3.3.8 Cytotoxicity Assay

The cytotoxicity of PPE-EA was evaluated in three different neural cell lines: PC12, NT2 and MCT17.2. PC12 cells were maintained in RPMI 1640 supplemented with 5% FBS

and 10% heat-inactivated horse serum, NT2 cells and C17.2 cells were maintained in DMEM supplemented with 10% FBS. The PEI treated group was used as a comparison. Cells were seeded in a 96-well plate at a density of $1x10^4$ cells per well with 100µl of complete medium. Cells were incubated in a 37^0C incubator for 24 hours, and then treated by adding 11µl serial diluted PPE-EA and PEI solution, which gave a final concentration ranging from 125µM to 1000uM. Following 24-hour incubation, 20µl of MTT solution (5mg/ml in PBS) was added, and the plates were incubated at 37^0C for another 4 hours. After that the un-reacted dye was removed from the plate, and 100ul of DMSO was added to each well. The optical density at 655nm in each well was measured by a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) using culture medium without cells as the blank. The relative cell viability (%) related to control cells cultured in media without polymer was calculated as [A] test/ [A] control×100.

3.3.9 Tissue biocompatibility

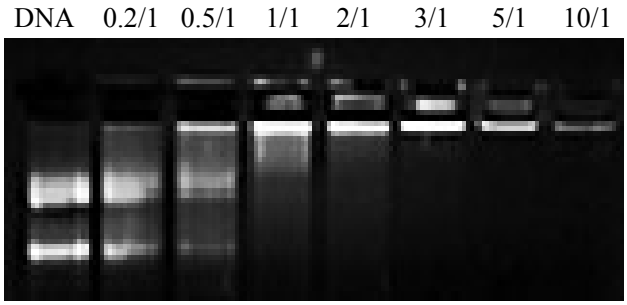
PPE-EA and PEI polymers (both with 100nmole charged group), and DNA complexed with the polymers in 5% glucose were injected into the mouse cisterna magna 7 days after intracisternal injection, animals were anesthetized by ether-air mixture and perfused transcardially with saline solution (20ml) followed by 30 mL of ice-cold fixative (4% paraformaldehyde, 0.05% glutaraldehyde in PBS). The whole brain was dissected out and fixed in phosphate buffered formalin (10%), and then embedded in paraffin. Tissue was cut at 8µm in thickness, and placed on gelatin coated slides, then stained by hematoxylin and eosin (H&E) for histological analysis.

For TUNEL staining, the tissue section was prepared in the same way as those for immunostaining assay. The slides were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4). After 1h of fixation at RT, the slides were rinsed with PBS and incubated with blocking solution (3% H_2O_2) for 10 min at RT. The slides were then incubated in a permeabilisation solution containing 0.1%Triton® X-100 in 0.1% sodium citrate for 2 min at $4.^0$C After rinsing slides twice with PBS, 50µl of TUNEL reaction solution from the TUNEL staining kit (Roche) was added to each sample. After 60 min incubation at 37^0C and washing, samples were analyzed under a fluorescence microscope. For the negative control, samples were incubated in 50µl of a label solution without terminal transferase.

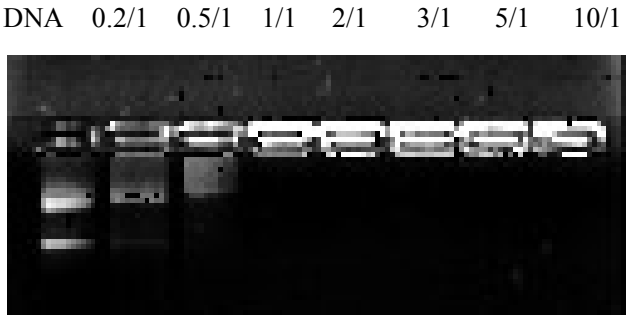
3.4 Results

3.4.1. Physical characteristics of PPE-EA/DNA complex

We first examined DNA binding and neutralization effects of PPE-EA using a gel retardation assay. At N/P ratios above 1, plasmid DNA cannot move out the gel slot, which implied that DNA was completely condensed into nanoparticles by PPE-EA or PEI (Fig.3-2).



DNA retardation by PEI (25KD)



DNA retardation by PPE-EA

Fig. 3-2. Agarose gel electrophoresis of polymer/DNA complexes. 0.2ug of plasmid DNA was complexed by PEI and PPE-EA at N/P ratio from 0 to 10.

We also examined sizes of PPE-EA/DNA particles and their stability in different solutions by light scattering (Table 1). The particles were prepared in 5% glucose, a solution that shows beneficial effects in formulating homogeneous PEI/DNA complexes and facilitating widespread diffusion of the complexes in the brain after injection into the cerebrospinal fluid (CSF) (Goula et al., 1998). The PPE-EA/DNA particles were then diluted with either 5% glucose (a low ionic strength condition) or 150mM NaCl (a high ionic, more physiological condition) before size measurement. Under the low ionic condition, the size of the PPE-EA/DNA particles changed with N/P ratios, increasing from 99 to 200 nm in the main particle populations as the N/P ratio changed from 0.5 to 2.

However, the particles were not stable in this 5% glucose solution. After 1-hour incubation, larger aggregates were formed, with diameters increasing to 1000 and 3000 nm at N/P ratios of 0.5 and 2, respectively. At high ionic strength, the complex size increased again with an increasing N/P ratio. The complexes tend to be larger, more uniform in size distribution, and less prone to aggregation (Table 1).

PEI/DNA complexes at N/P ratio of 15/1, the optimized N/P ratio for gene delivery to the brain in our hands, were also included for comparison (Table 1). The complexes obtained were smaller in the 5% glucose solution, around 90 nm as opposed to 120 nm in the 150 mM NaCl solution. Aggregation occurred in both solutions with time, with approximately 50% of the complexes displaying a size around 400 nm after 1-hour incubation. The AFM figure confirmed this observation (Fig.3-3).

Table 2: DNA-polymer complex size (nm)*

A: 5% Glucose

	Peak I		Peak II		Peak III	
	Mean±SD	%	Mean±SD	%	Mean±SD	%
PPE-EA(N/P=0.5)						
0h	98.6 ± 22.8	58.8	433.6 ± 100.4	17.8	979.8 ± 145.9	16.7
1h	163.3 ± 28.9	60.4	0	0	932.1 ± 151.8	33.6
PPE-EA(N/P=2)						
0h	200.4 ± 45.5	51.5	1004.3 ± 131.9	19.3	3000 ± 393.3	29.0
1h	0	0	0	0	3000 ± 299.6	96.0
PEI(N/P=15)						
0h	86.9 ± 31.1	82.9	0	0	0	0
1h	137.7 ± 32.0	63.2	488.0 ± 58.9	26.9	0	0

B: 150mM NaCl

	Peak I		Peak II		Peak III	
	Mean±SD	%	Mean±SD	%	Mean±SD	%
PPE-EA(N/P=0.5)						
0h	152.3 ± 27.9	78.2	0	0	0	0
1h	171.6 ± 20.8	70.0	0	0	0	0
PPE-EA(N/P=2)						
0h	557.8 ± 164.1	100	0	0	0	0
1h	568.1 ± 153.9	100	0	0	0	0
PEI(N/P=15)						
0h	118.0 ± 35.1	65.2	241.9 ± 49.6	34.8	0	0
1h	121.5 ± 14.9	54.0	375.2 ± 94.7	24.6	482.1 ± 153.0	16.1

* Ten micrograms pCAG-luc in 50 μl 5% glucose were complexed with polymers in 50 μl 5% glucose. The complexes were diluted either in 5% glucose (A) or 150mM NaCl (B) to 1 ml for the measurement of particle sizes with N4 Plus Submicron Particles Sizer (COULTER, USA) at room temperature. Scattering light was detected at 90^0 angle, running for 200 sec for each sample (n = 8 for each preparation), and analyzed in the Unimodel Analysis mode.

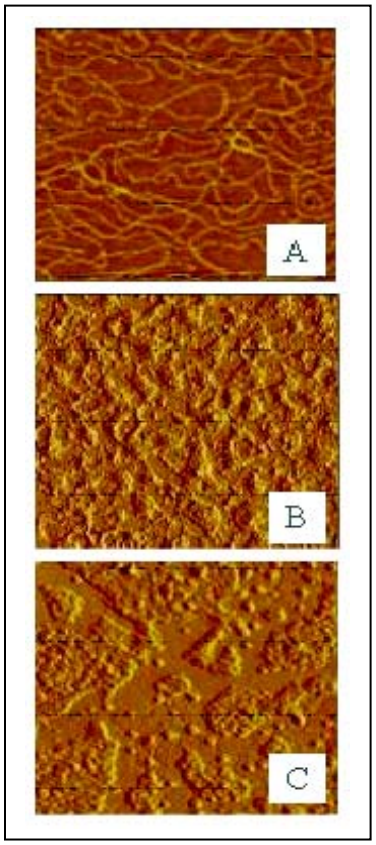


Fig. 3-3. AFM images. A: Plasmid DNA; B: PPE-EA/DNA at N/P ratio of 0.5/1; C: PPE-EA/DNA at N/P ratio of 2/1.

3.4.2 Gene transfection efficiency

The feasibility of using PPE-EA/DNA complexes for CNS gene delivery was then examined in mice after intracisternal injection using luciferase as a reporter gene. PPE-EA/DNA complexes at different N/P ratios from 0 to 10 were tested. Gene transfer efficiency increased with N/P ratio from 0.2 to 2, and then dropped as the N/P ratio reached 10 (Fig.3-4).

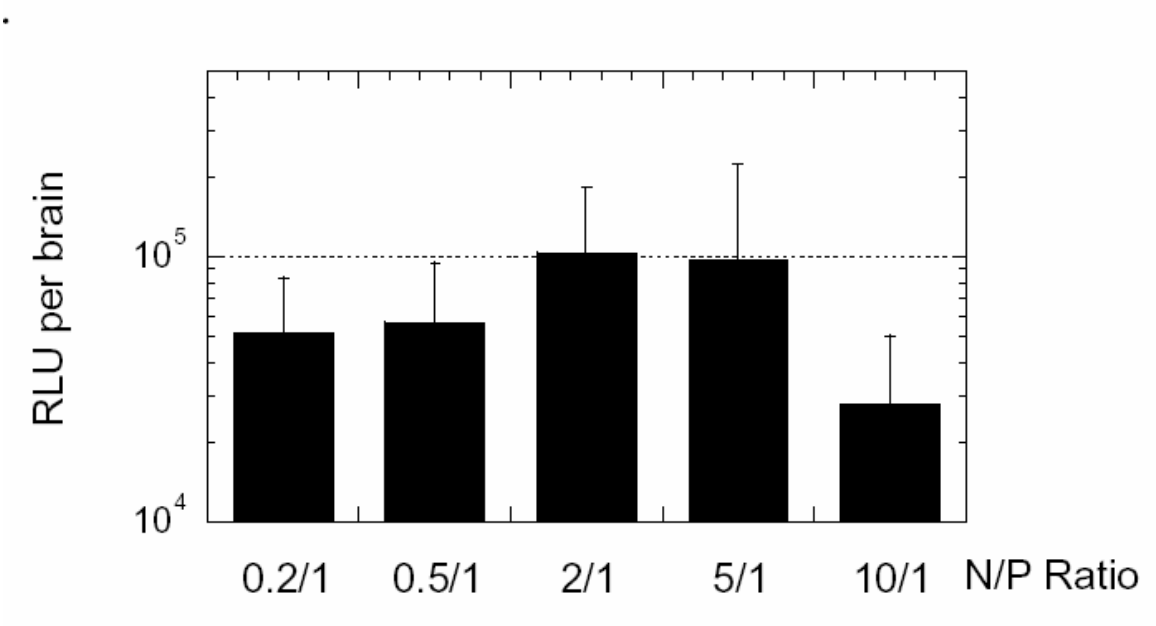


Fig. 3-4. Luciferase expression in mouse brain after intracisternal injections of PPE-EA/pCAG-Luc complexes with various N/P ratios. Values are presented as means ± standard deviation(n=5).

To compare the gene delivery efficiencies between PPE-EA/DNA complexes and PEI/DNA complexes, as well as naked DNA, a time course study was carried out. PPE-EA/DNA complexes with N/P ratios of 0.5 and 2, naked DNA and PEI/DNA complexes with N/P ratio of 15 (the optimized N/P ratio for brain gene delivery of PEI in our hands) were used (Fig.3-5). One day after injection, PEI/DNA showed the highest transgenic expression at about 2 x 10^5 RLU (relative luciferase unit) per brain, about 4 folds higher than that of PPE-EA/DNA at N/P of 2 and 2 folds higher than that of Naked DNA and PPE-EA/DNA at N/P of 0.5. Three days after injection, the luciferase expression level

mediated by PPE-EA/DNA at N/P of 2 increased to 1.8 x 10^5 RLU/mouse, whilst the expression levels of other three groups maintained at day one's level. Ten days after injection, the expression level for PPE-EA/DNA at N/P of 2 was about the same as day 3, while other expression levels started to drop. Until 28 days after injection, PPE-EA/DNA at N/P of 2 still provided a similar level of transgenic expression as previous time points at about 8 x10^5 RLU/brain, which was significantly higher than those provided by PEI/DNA, naked DNA and PPE-EA/DNA at N/P of 0.5.

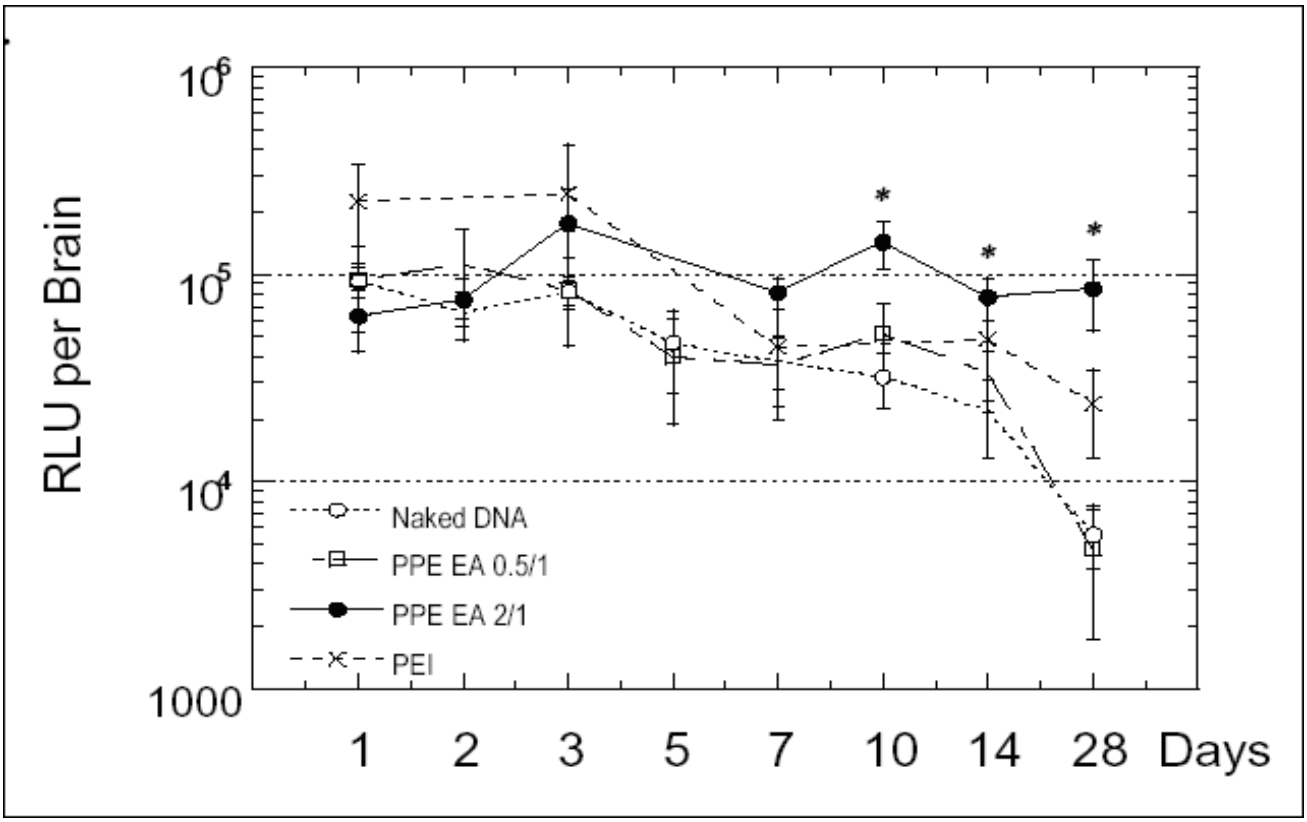


Fig. 3-5. Time course for Naked DNA and PPE-EA/DNA complexes (N/P=0.5, 2.0) after intracisternal injection. Naked DNA and PPE-EA/DNA complexes (N/P=0.5, 2.0) were given at a dose of 1ug of DNA per mouse in 5% glucose at a total volume of 1ul. The value represent the average RLU of the whole brain of 6 mice. Means ± standard deviation (n=6). Asterisk denotes significantly different from the control Naked DNA, PEI/DNA and PPE-EA/DNA (N/P=0.5) ($p<0.05$).

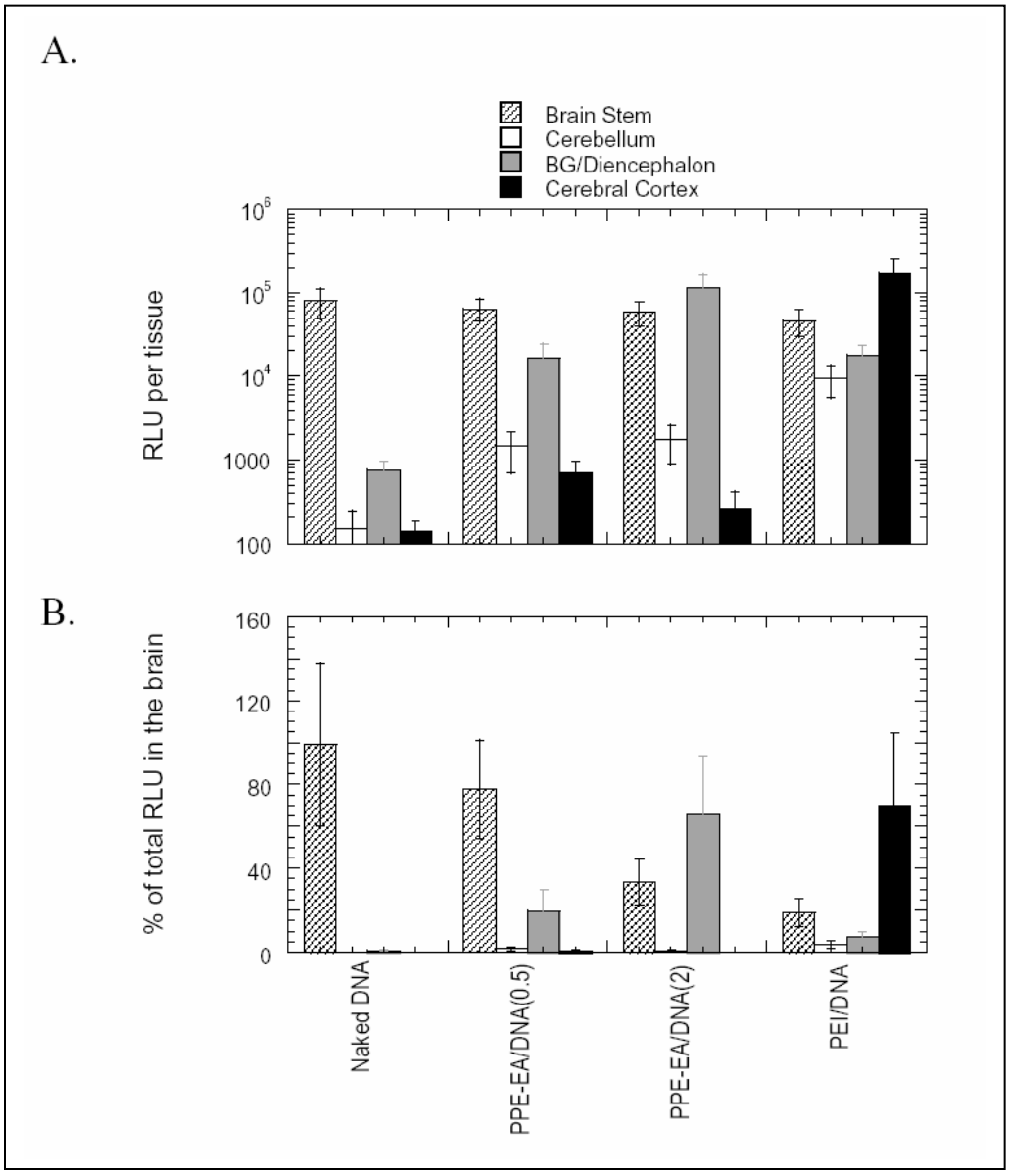


Fig. 3-6. Comparison of the distribution of reporter gene expression with various gene delivery systems. Samples were collected at 3 days after injection. A: The luciferase expression level of each brain area was presented; B: The percentage of luciferase expression in each brain area was presented. Six mice per group were used. Bars represent means ± SE.

To compare the distribution of the transgene expression, the brain samples collected 3 days after injection were dissected into four parts and analyzed for luciferase activity:

brain stem, cerebellum, basal ganglia/diencephalons and cerebral cortex (Fig. 3-6). Almost all the luciferase activities mediated by naked DNA delivery were located in the brain stem. A similar transgene expression pattern was observed with PPE-EA/DNA complexes at N/P ratio of 0.5. With PPE-EA/DNA complexes at N/P ratio of 2, significant levels of luciferase expression were detected both in the brain stem and the basal ganglia/diencephalons, accounting for about 33 % and 65 % of the total activity, respectively. In contrast, PEI/DNA complexes appeared to have diffused to the cerebral cortex significantly, with 70 % of the luciferase activity being found there. All four groups showed very low levels of transgene expression in the cerebellum.

Immunostaining of the brain sections using an antibody against luciferase supported the distribution profiles of gene expression described above for the PPE-EA/DNA complexes at a N/P ratio of 2. Strong signals were observed near the injection site, mainly in the meninges (Fig.3-7A). The meninges of the brain regions away from the injection site were also positively stained (Fig. 3-7B). For the PEI/DNA complexes, the luciferase was clearly stained even in the meninges of the cerebral cortex (Fig. 3-7C). Gene expression in the parenchyma of the brain was also examined 3 days after intrastriatum, instead of intracisternal, injection of PPE-EA/DNA complexes at a N/P ratio of 2. Many cells close to the injection track were luciferase-positive, several of which displayed typical neuronal morphology (Fig. 3-7D).

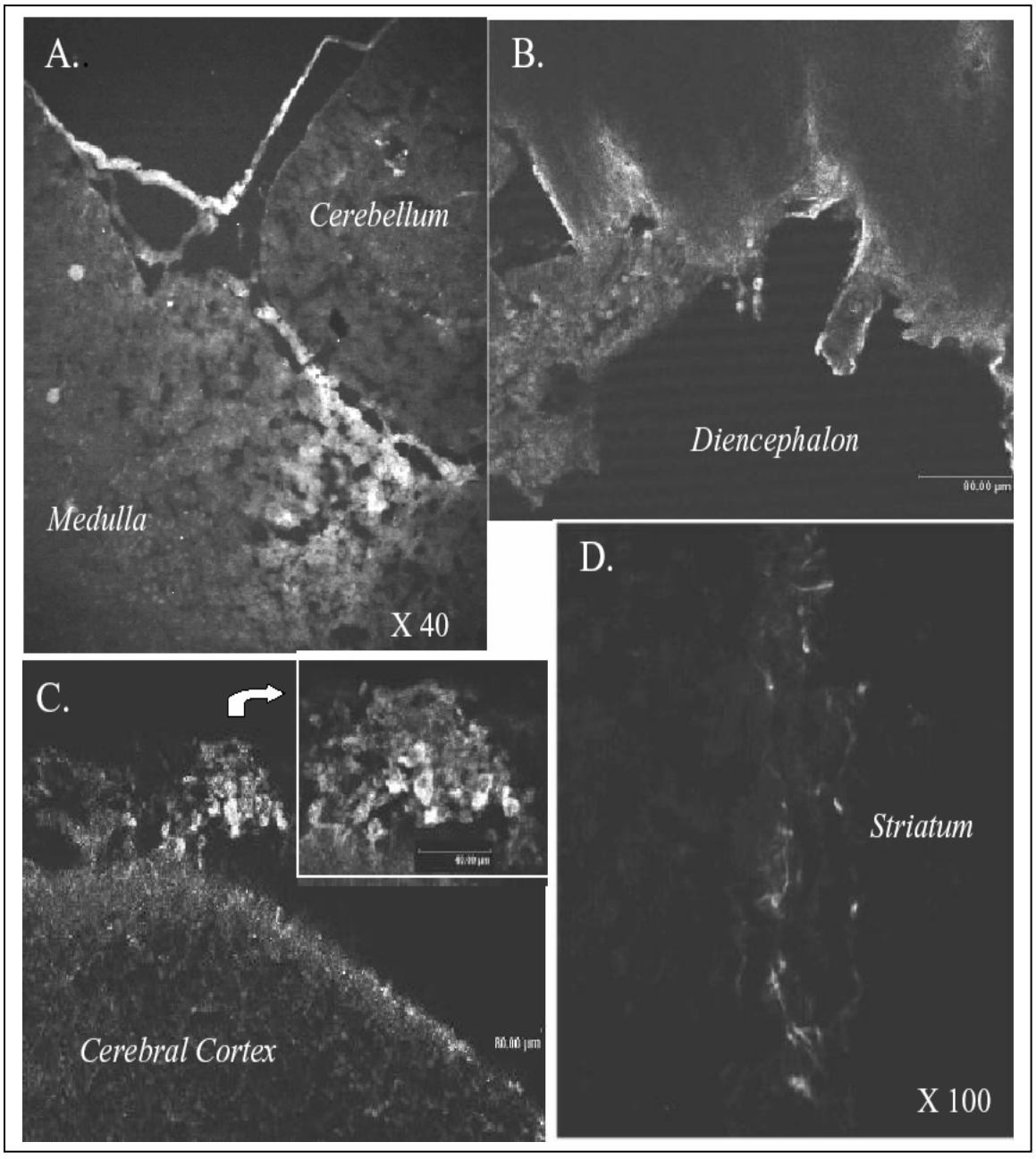


Fig. 3-7. Confocal images of luciferase expression in brain tissues. Immunostaining with an antibody against luciferase 3 days after intracisternal (a–c) or intrastriatum (d) injection of 1 mg of pCAG-Luc complexed with PPE-EA at an N/P ratio of 2 (a, b, d, bar: 80 mm) or with PEI at an N/P ratio of 15 (c, bar: 80 mm). The inset in (c) shows positively stained cells in arachnoid granulations (bar: 40 mm).

3.4.3. Cytotoxicity and tissue responses

Knowing that some cationic polymers, like PEI and poly-L-lysine, exhibit cytotoxicity,(Godbey et al., 2002) we tested the cytotoxicity of PPE-EA in three different neural cell lines. C17.2 is a multipotent cell line generated via retrovirus-mediated V-myc transfer into murine cerebellar progenitor cells. PC12 is a cell line isolated from rat pheochromocytoma, which is inducible to exhibit a neuronal phenotype by never growth factor. NT2 cell is a human cell line exhibiting characteristics of committed CNS neuronal precursor cells. Among the three cell lines tested, C17.2 was the most robust, maintaining 90% viability at 1000 μM of PPE-EA, while only 50 to 70% of PC12 and NT2 cells were viable at 500 μM (Fig. 3-8). All cell lines were more vulnerable to the PEI treatment. For example, at 1000 μM, cell viability ranged from 40 to 90% in the PPE-EA treated groups, whilst most of cells died in the PEI-treated groups. After neuronal differentiation, C17.2 showed even higher susceptibility to PEI induced death, most of which died at a concentration as low as 165 μM, while close to 80% of the C17.2 neurons were still viable at 1000 μM of PPE-EA (Fig. 3-8B).

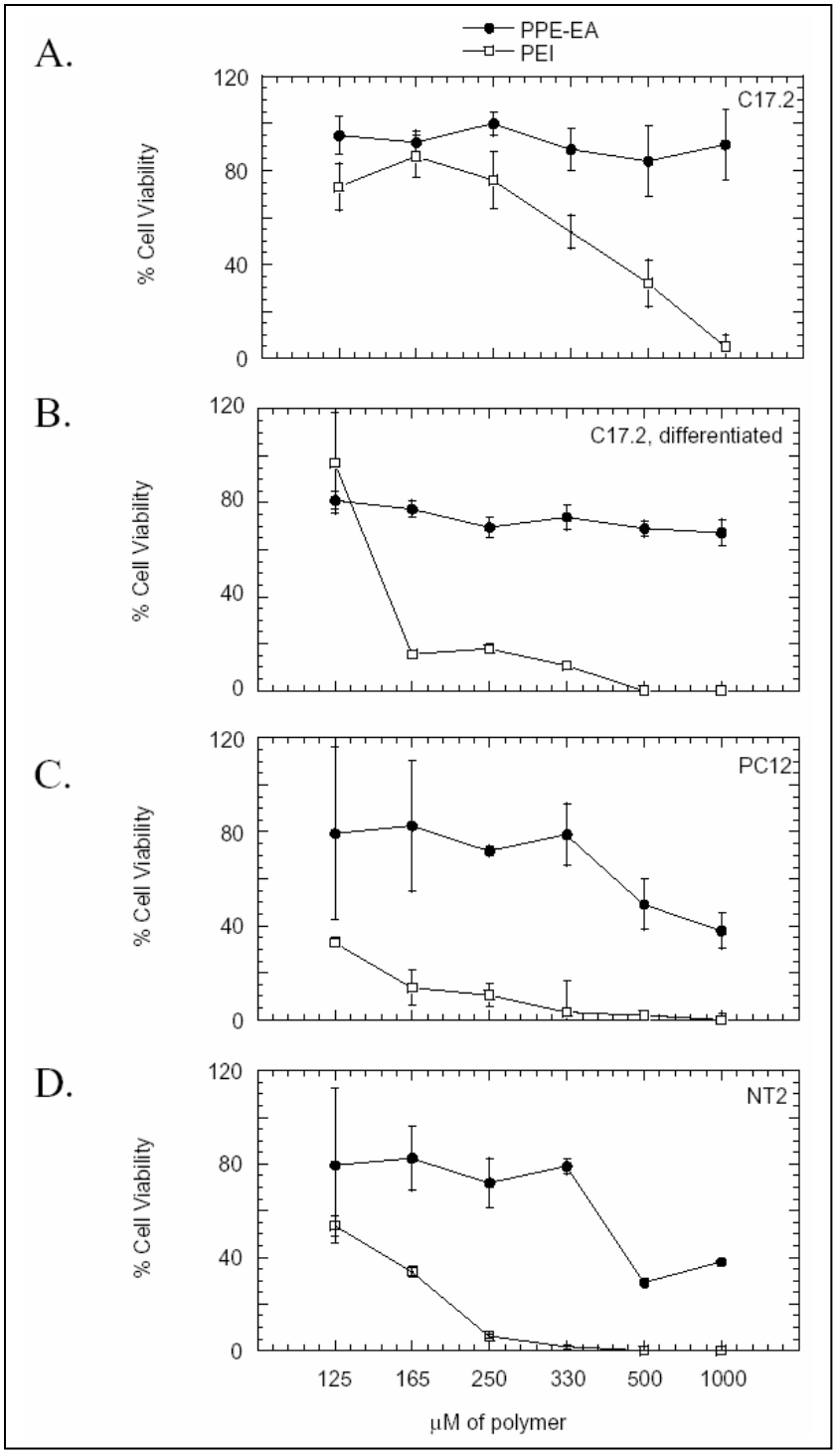


Fig. 3-8. Viability assay in C17.2 (A: undifferentiated, B: differentiated), PC12 (C), and NT2 (D) cells.

To further investigate their biocompatibility, PPE-EA and PEI polymers were intracisternally injected into mice cisterna magna at the same doses of charged groups (100 nmole of amino group for both PPE-EA and PEI) and the tissues were collected 7 days later for histological analysis. No significant inflammatory reactions were observed by H&E staining in both PPE-EA (Fig. 3-9A) and PEI (Fig. 3-9B) treated animals. Effects of the polymer/DNA complexes were also examined. Consistent with what we observed before, (Shi et al., 2003) TUNEL staining revealed positively stained cells in the PEI/DNA particle treated group (Fig.3-9D). No such cells were detectable in the PPE-EA/DNA complex treated animals (Fig.3-9C).

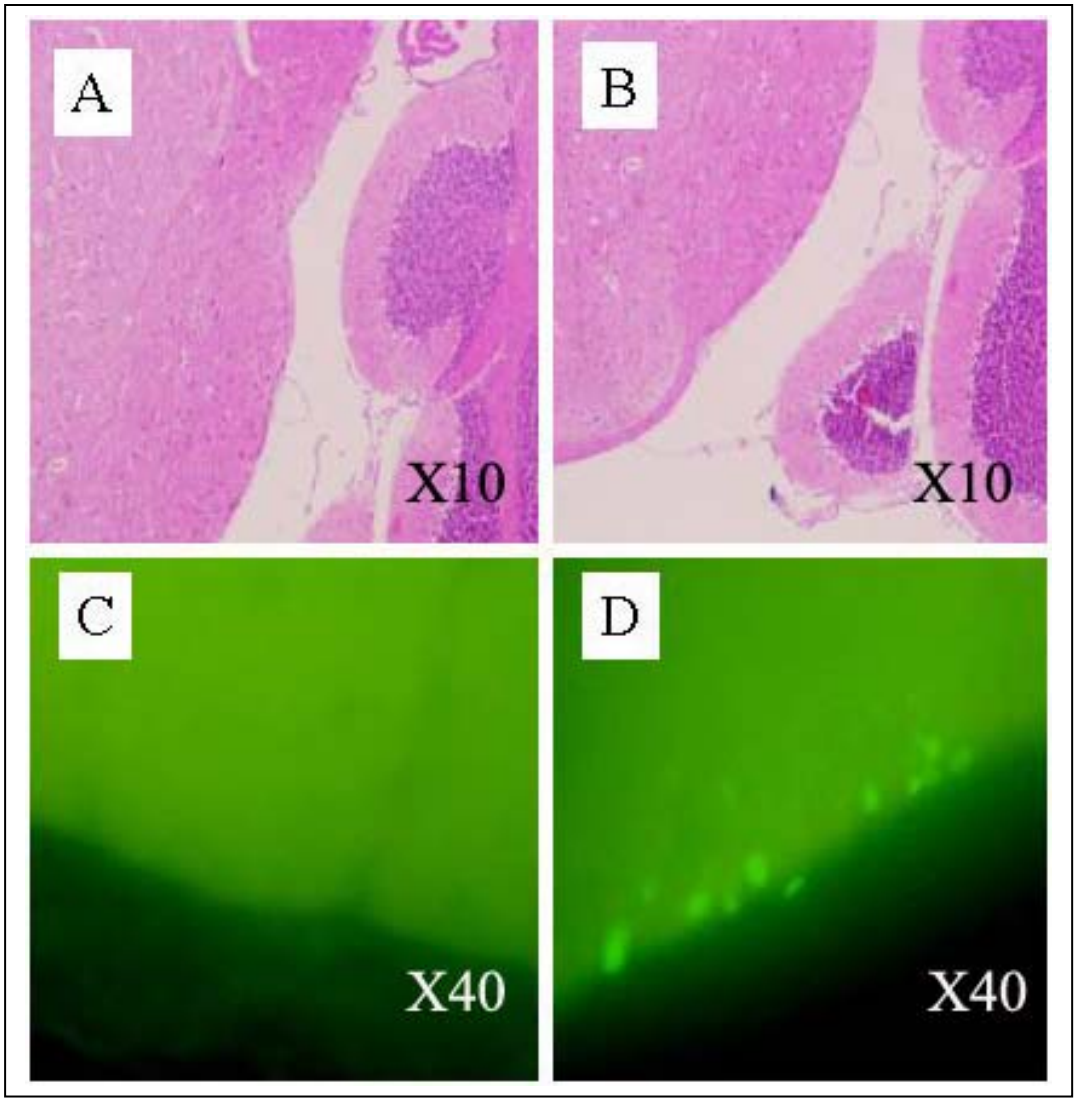


Fig. 3-9. Tissue response at day 7 after intracisternal injection of PPE-EA, PEI and their DNA complexes. (A, B) H&E-stained brain stem/cerebellum tissue sections from animals intracisternally injected with PPEEA (A) and PEI (B); (C, D) TUNEL stained tissue sections from animals intracisternally injected with 1 mg of DNA complexed with of PPE-EA (C) or PEI (D). Note TUNEL-positive cells in (D). The original photographs were taken at _40 magnification for (A) and (B) and _200 magnification for (C) and (D).

3.5 Discussion

PPE-EA is a recently developed water-soluble and biodegradable polyphosphoester with positively charged side chains.(Wang et al., 2002) PPE-EA condenses DNA efficiently and protects it against nuclease and serum degradation. (Wang et al., 2001) A unique feature of the PPE-EA gene delivery system is controlled release of plasmid from the polymer/DNA complexes, achieved as a consequence of polymer degradation. The released DNA retains structural and functional integrity and may subsequently be taken up by cells to mediate gene expression.(Wang et al., 2002) The release rate is adjustable by varying the charge ratio of PPE-EA to DNA. At an N/P ratio of 0.5, complete release of DNA is achieved within 3 days in vitro. Higher charge ratios lead to slower release of DNA from the polymer DNA complexes. It may take 3 days for the onset of DNA release from the complexes at N/P ratio of 2 to occur. (Wang et al., 2001) This explains a slow increase of gene expression levels during the first 3 days after the injection of the complexes reported here. Complete release of DNA from complexes with a higher N/P ratio takes much longer, most likely because of the stronger grip on the DNA with the increased amount of the polymer in the complexes. This may serve as one mechanism underlying the prolonged gene expression observed in this study. The CSF can only accommodate a small injection volume, such as the one reported here. To provide a high enough DNA dose, a high concentrations of DNA complexes has to be used. This would probably lead to an even slower DNA release. Furthermore, the larger aggregates of PPE-EA/DNA particles formed in physiological fluids would have difficulty to be transported from the CSF compartment into the superior sagittal sinus via absorption across the arachnoid granulations and villi. The retention of the biodegradable aggregates along the

meningeal surface may facilitate sustained release of plasmid DNA in the CSF, prolonging the subependymal expression of the transgene.

Large aggregates of PEI DNA complexes display acute toxicity after tail vein injection and may kill 50% of the animals within 30 min. (Hecker et al., 2001) After CSF injection, the aggregates are one possible factor causing cell death in the meninges. (Shi, Tang, Gao, Ma, Liu, Li, Zeng, Ng, Leong, and Wang 2003) PPE-EA/DNA complexes also form aggregates at N/P ratio of 2, but display lower toxicity in neural cells and no apoptotic induction effects after injection into the rat CSF. PPE-EA is designed to have nontoxic building blocks and would ultimately be hydrolyzed within weeks into α-propylene glycol, phosphate and ethanolamine that have minimal toxicity profiles. (Wang et al., 2002) The biocompatibility of the polymer raises the possibility of repeated injection to achieve an even longer period of gene expression. Transient expression of plasmid DNA is a disadvantage of nonviral gene delivery systems in treating many of the neurological disorders, because of their slow pathogenesis. In an attempt to achieve prolonged transgene expression through repeated injection of PEI/DNA complexes into the CSF, we observed a 70% attenuation of gene expression following re-dosing at a 2-week interval. (Shi et al., 2003) Using a PPE-EA instead of PEI in this kind of administration, one would expect a long-term gene expression at a significantly higher level.

The intrathecal injection approach adopted in this study bypasses the blood-brain barrier by delivering reagents directly into the CSF. In other studies, direct injection into the CSF via the lateral ventricles of the rat brain, DNA complexed by cationic liposome(Driesse,

et al., 2000) and recombinant adenovirus vectors(Reijneveld, Taphoorn et al. 1999) may mediate transgene expression throughout the entire brain. Given the small size of the mouse model, intracisternal injection is more practical than other intrathecal injection methods. Delicate techniques of intracisternal injection were exploited for inducing leptomeningeal metastases or delivering drugs, (Nguyen et al., 2000) but seldom used for gene delivery. In this study intracisternal gene delivery allowed widespread gene expression in the mouse brain, although the distribution pattern was somewhat vehicle-dependent. PEI/DNA complexes produced the most widespread dispersion, followed by PPE-EA/DNA complexes. Naked DNA, probably because of its vulnerability to DNAses, may not be able to diffuse intact far from the injection side. The difference in the distribution of gene expression between PPE-EA/DNA complexes at N/P ratios of 2 and 0.5 is probably attributed to the fact that at the low N/P ratio a significant portion of the DNA is dissociated from the complexes after injection. PEI/DNA complexes used in this study, which were prepared at N/P ratio of 15, provided a high level of gene expression in the cerebral cortex, a region far away from the injection site. These findings suggest that a higher positive surface charge may facilitate widespread distribution of polymer/DNA complexes through the CSF.

In summary, the present study has demonstrated that PPE-EA may mediate gene transfer in the CNS, with efficiency superior to naked DNA and, in terms of prolonged gene expression, to PEI. Unlike PEI, PPE-EA is biodegradable and displays low toxicity in the nervous system. To our knowledge, this study is the first one using a controlled DNA

release system in the CNS. The results establish PPE-EA as a promising gene carrier applicable to the CNS.

CHARPTER 4. NEURON-TARGETED GENE TRANSFER BY BACULOVIRUS-DERIVED VECTOR ACCOMMODATING A NEURON-SPECIFIC PROMOTER

4.1 Abstract

Recombinant baculovirus vectors have been adopted as potent gene delivery vectors for mammals in recent years. But the poor transfection efficiency in neurons reported in the previous studies hindered their potential in gene therapy of neurodegenerative diseases where neurons are the main targeted cell group. The aim of this study is to develop a new recombinant baculovirus vector which is capable of mediating neuron-targeted and persistent gene expression in CNS. To realize this purpose, a novel baculovirus vector, namely BV-CMV E/PDGF, was constructed by adopting a hybrid neuron-specific promoter to drive the reporter gene expression. The results indicate that the neuronal, but not a viral, promoter successfully modified the baculovirus vector with neuron-targeted gene expression in both in vivo and in vitro experiments. These results suggest that recombinant baculovirus may be a promising vector to transfer genetic materials into neurons for gene therapy of neurodegenerative diseases.

4.2 Introduction

The use of baculovirus *Autographa californica* multiple nuclear polyhedrosis viruses (AcMNPV) as mammalian cell expression vector has recently been viewed as a new generation of gene therapy vehicles with great promise (Ghosh et al., 2002;Kost and Condreay 2002). Baculovirus has some inherent advantages, such as large insert capacity, simple manipulating procedure, and replication deficiency in mammalian cells, circumventing the potential risks triggered by virus replication. The viruses produce little to no microscopically observable cytotoxcity, owing to its low immunogenicity in mammals. A broad spectrum of vertebrate cell lines has been successfully transfected by baculovirus (Ghosh et al., 2002; Kost and Condreay 2002), including several human primary cells (Boyce and Bucher 1996;Dwarakanath et al., 2001;Hofmann et al., 1995;Sarkis et al., 2000). In vivo studies demonstrated the transgene expression mediated by the baculovirus vectors in the liver (Ni 2001a;Sandig et al., 1996), skeletal muscle (Pieroni et al., 2001), pancreas (Ma et al., 2000), brain (Lehtolainen et al., 2002b;Sarkis, et al., 2000), and carotid artery (Airenne et al., 2000), with expression levels comparable to those mediated by adenovirus vectors in some organs (Airenne et al., 2000;Lehtolainen et al., 2002b).

Baculovirus vector-mediated gene transfection has been investigated in the nervous system. The original reports described an efficient transduction of neural cells *in vitro* and *in vivo* by baculoviurs vectors containing a CMV promoter (Sarkis et al., 2000). In primary cell cultures of human embryonic brains, neuroepithelial, neuroblastic, and glial cells could be infected. In vivo studies using adult nude mice demonstrated that mainly

astrocytes, with only a few neurons, were transduced in baculovirus vector injected-brains. Lehtolainen et al (2002b) then carefully examined the cell-type specificity of baculovirus-mediated gene expression in the brain (Lehtolainen et al., 2002b). Baculovirus vectors containing a CMV promoter were injected into rat corpus callosum. Cuboidal epithelial cells of the choroids plexus were identified as the main target cells of the viruses in the brain. However, only modest gene expression was detected in endothelial cells and very limited or no expression was found in other types of brain cells, including neurons and astrocytes.

The major functional cells in the nervous system are neurons, which carries out the fundamental task in receiving, conducting and transmitting signals. Therapeutic protection of these cells is one of the main goals of molecular therapy of neurological disorders. Examples include protecting dopaminergic nigrostriatal neurons in Parkinson's disease (Duvoisin 1992), entorhinodentate neurons in Alzheimer's disease (Gomez-Isla et al.，1996), and spinal motoneurons in amyotrophic lateral sclerosis. To overcome the limitation of using baculovirus vectors in the transduction of neurons, we set out to use a previously developed strong neuronal specific promoter, CMV E/PDGF, to hopefully improve transgene expression in neurons.

CMV E/PDGF is a hybrid neuronal specific promoter previously developed in our laboratory (Liu et al., 2004). This promoter was constructed by appending a 380-bp fragment of the CMV enhancer 5' to the PDGF-β promoter (Fig 4-1). The promoter for platelet-derived growth factor (PDGF) B-chain (PDGF-β promoter) is one such cellular

promoter that may offer specific gene expression in neurons. PDGF β-chain is heavily expressed in neurons throughout the brain and the spinal cord, but not in glial cells （Sasahara M et al., 1991）. The expression efficiency and neuronal specificity of this promoter was proved in vitro and in vivo using plasmid DNA by Dr. Liu Beihui, which presented preserved neuron-specificity of the PDGF-β promoter as well as improved transfection efficiency after the CMV enhancer sequence being inserted (Liu et al., 2004).

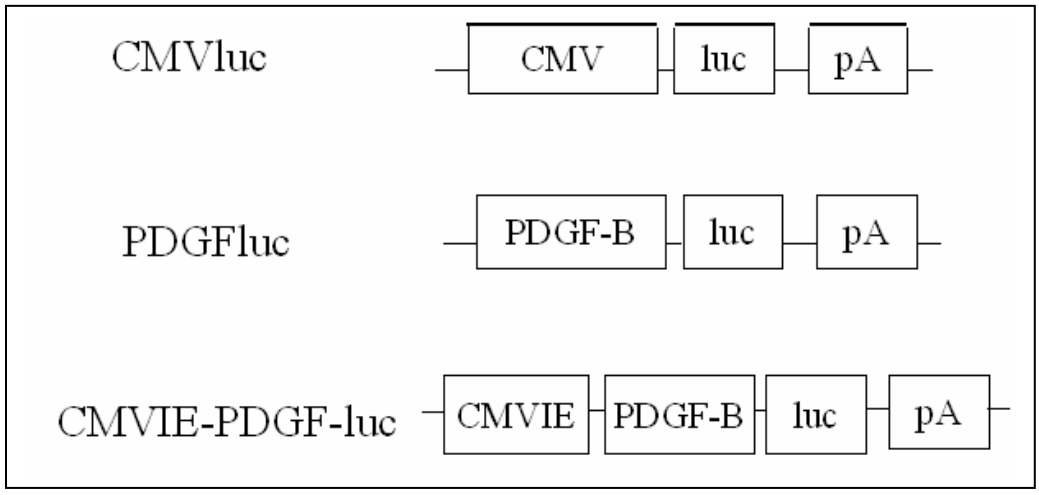


Fig. 4-1. Schematic pictures of expression cassettes with different promoters

The function of the new recombinant vector, namely BV-CMV E/PDGF, was then tested both in primary neural cell cultures and in rat brain. Another two vectors, BV-CMV (accommodating a CMV promoter) and BV-PDGF (accommodating a PDGF promoter), were constructed and used as the controls. The neuronal specificity of BV-CMV E/PDGF was examined by infection of primary neural cells and injection into the rat striatum. After direct injection into the rat striatum, long-term transgene expression at relatively high level up to 4 weeks was found for BV-CMV E/PDGF, while only transiently high expression within 3 days was detected for BV-CMV. These results indicate that BV-

CMV E/PDGF may be a suitable vector for gene therapy of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, where prolonged gene delivery to neurons is essentail.

4.3 Materials and methods

4.3.1 Production of recombinant virus vectors

Briefly, recombinant baculovirus vectors were constructed according to the manual of Bac-To-Bac Baculovirus Expression system (Invitrogen, Life Technologies, USA). Luciferase cDNA under the control of CMV E/PDGF, CMV, or PDGF promoter was inserted into the transfer plasmid pFastBac1 (Fig. 4-2.). Promoters were inserted between NotI & XbaI sites, respectively, and luciferase cDNA was inserted into the pFASTBac1 between XhoI & HindIII sites downstream to the promoters. Recombinant baculovirus particles were propagated in Sf9 insect cells following standard methods (Fig. 4-3.) (O'Reilly, Miller et al. 1992). Budded virus from insect cell culture medium was filtered through a 0.2-*u*m pore size filter (Millipore, USA) and concentrated by ultracentrifugation at 28,000 g for 60 min. The viral pellet was re-suspended in phosphate-buffered saline (PBS) and infectious titers were determined by a plaque assay using Sf9 cells (Fig. 4-4).

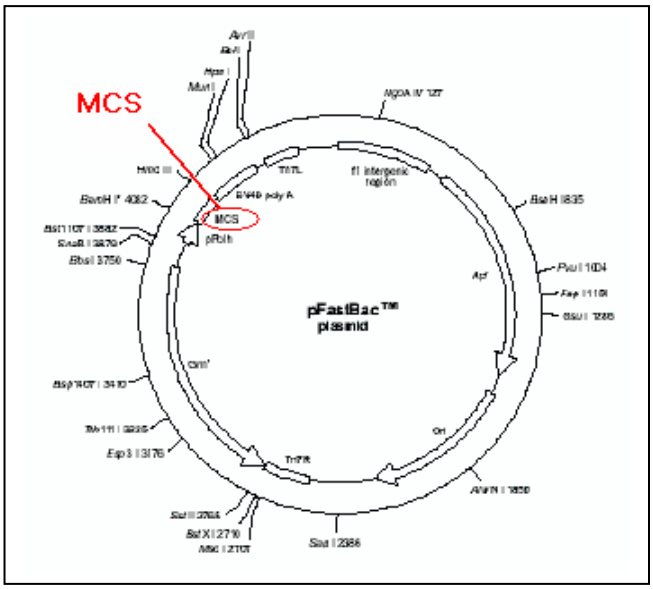


Fig. 4-2. X Map of pFastBac^{TM} plasmid (adapted from manual of Invitrogen)

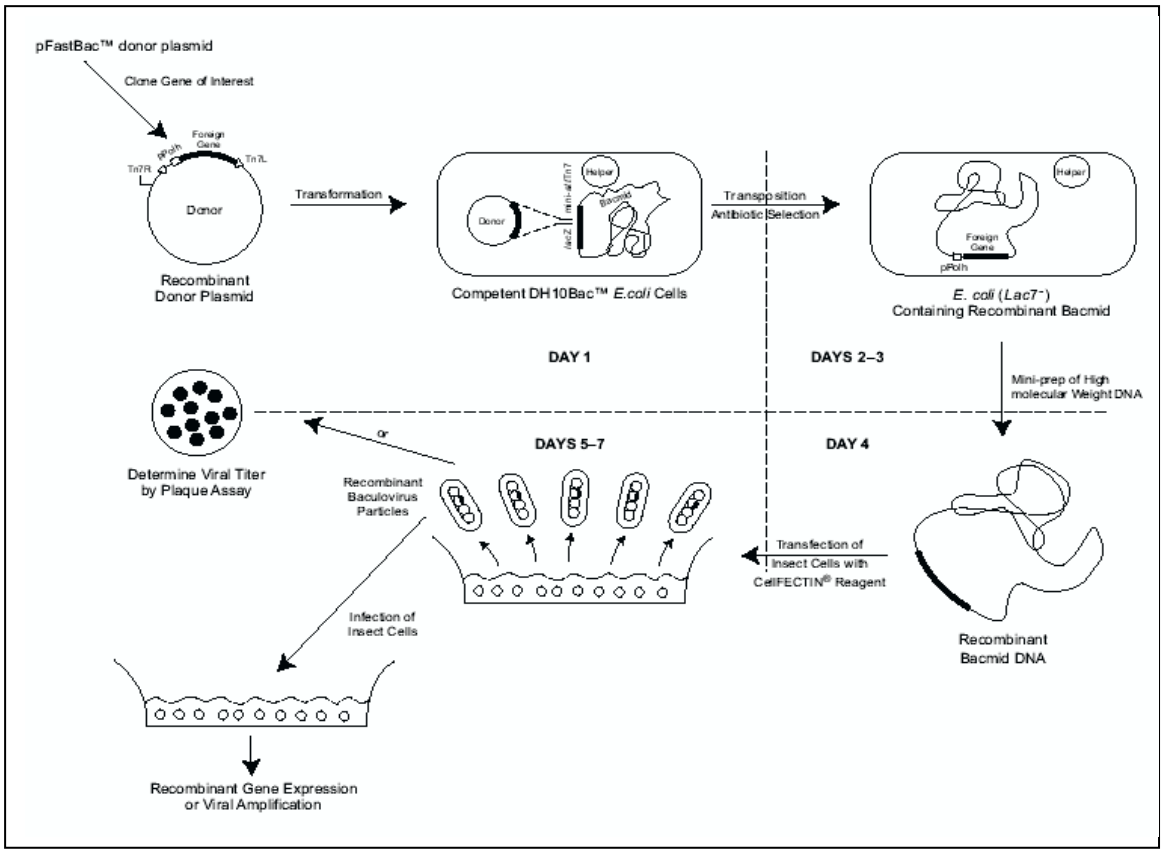


Fig. 4-3. Procedure of recombinant baculovirus particle generation (adapted from manual of Invitrogen)

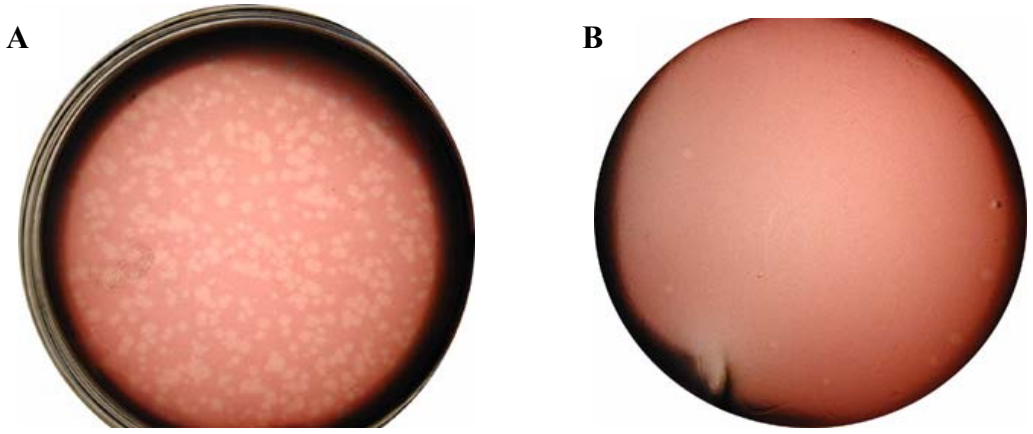


Fig. 4-4. Measurement of viral titer by plaque assay using Sf9 cells. Plaques were observed as A: 10^6 dilution of virus stock; B: 10^8 dilution of virus stock.

4.3.2 Cy3 labeling of baculovirus

Purified virus was labeled with carbocyanine dyes Cy3 (Amersham). Baculovirus stocks to be labeled were adjusted to a concentration of $2x10^{10}$ particles/ml in sodium bicarbonate buffer, pH 9.3. Cy3 reagent was previously reconstituted in 1 ml of 0.1 M sodium carbonate, pH 9.3 (designed to label 1 mg protein to a final molar dye/protein ratio between 4 and 12). 100µl of virus preparation was mix with 200µl of the Cy3 reagent. After 30 min at room temperature, labeled virus was purified by centrifugation at 28,000 g for 30min. The viral pellet was washed in 1xPBS for 2 times to remove unreacted Cy3, and then re-suspended in 1x PBS. The purified virus was aliquoted into single-use vials and stored at 4°C prior to use.

4.3.3 Cell line and primary cell cultures

PC12 cell culture: The *in vitro* transfection experiment was performed in differentiated PC12 cells. PC12 rat pheochromocytoma cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% horse serum. For microscopy, cells were seeded onto 8-well chambered tissue culture treated glass slides (Falcon) at a density of $2x10^4$ cells per chamber. The cells were induced to differentiate in 50 ng NGF per milliliter medium for 3-4 days before viral transfection.

Primary neuronal cell culture: Primary neuronal cell cultures were established from the cortices of embryonic Wistar rats at gestational day 20, according to the protocol described by Pongrac and Rylett (Pongrac and Rylett 1998). Cortices free from meninges were dissected and minced into small pieces. Then, these tissues were triturated into

individual cells in 10% FBS supplemented Dulbecco's modified Eagle's medium (DMEM) by a 9-inch fire-polished Pasteur pipette with successively smaller bore. Cell suspensions were diluted with 10% FBS supplemented DMEM and transferred to a centrifuge tube to let the chunks settle down. The supernatants were then collected and centrifuged at 900 rpm for 5 minutes. The cell pellet was re-suspended gently in 10% FBS supplemented DMEM, and cell viability was checked by trypan blue staining prior to plating. Cells were plated into the wells of 8-well chambered tissue culture treated glass slides at a density of 7.5 x 10^5 viable cells per cm^2. Chambered glasses were coated with one layer of poly-l-lysine (Sigma; 0.05 mg/ml; 1 h) on the bottom and another layer of laminin (Sigma; 0.02mg/ml; 1h) on the surface prior to use. Cells were cultured in serum-supplemented medium for 2 hrs to initiate attachment of neurons to the substratum, and to inactivate proteolytic enzymes released from damaged cells during trituration. After the initial attachment, medium and unattached cells were removed and replaced by serum-free DMEM/F12 media supplemented with 1% N2 (Gibco). The cells were then incubated at 37°C in a humidified atmosphere with 5% CO_2. Medium was changed every two to three days by replacing half of the conditioned medium with fresh medium. After 5 days of culture, the neuronal cells were used for baculovirus infection. This part of work was done by Ms. Ma Yuexia.

Primary glial cells culture: Primary glial cell cultures were also established from the cortices of embryonic Wistar rats at gestational day 20. Individual cells were collected as described above. The cells were plated at a density of 4 $\times 10^5$ viable cells/cm^2 on poly-l-lysine/laminin coated dishes, and were grown to confluency in DMEM/F12 medium supplemented with 10% FCS. 100% confluent cells were partially digested by trypsin to

remove the neurons growing on the surface of the glia cells. After at least 14 days of culture, the cells were detached and plated into 48-well culture plate at a density of 2.5 $\times 10^4$ cells per well 24 hrs before virus inoculation.

Mixed primary neural cell culture: Mixed primary cell culture including both neurons and glial cells was initiated the same way as described for primary neuron culture. Cells were seeded in the coated chamber at a lower density of 3.5 x 10^5 viable cells per cm^2 and cultured in DMEM/F12 medium supplemented with 10% FCS and 1% N2. The cells were then incubated at 37°C with 5% CO_2 in a humidified atmosphere. Half of the culture medium was replaced by fresh medium every 2-3 days. After 10 days, the primary neuron and glia cells mixtures were used for viral transfection studies.

4.3.4 Virus infections

Cells were infected by Cy3 labeled or unlabeled baculovirus in Opti-MEM (Invitrogen, Netherlands) at 37^oC. The time course of viral exposure was about 1 hour unless otherwise noted. After the virus-containing medium was removed and the cells were fed with fresh medium and maintained at 37°C for additional incubation periods. Cells were collected at different time points as needed. For cells infected by Cy3 labeled virus, culture medium was removed and the cells were washed three times with warm PBS (GIBCO, Grand Island, N.Y.) to remove the free viral particles prior to fixation. Cells were fixed with 4% paraformaldehyde in PBS for 5-10 min at 37^0C and washed three times with PBS before they were used for immunostaining and confocal microscopy. For

luciferase expression experiments performed in mixed primary neural cells, double immunostaining was performed.

4.3.5 Animals

Adult male Wistar rats (weighing 250-320g) were used in this study. In the handling and care of all animals, the International Guiding Principles for Animal Research as stipulated by World Health Organization (1985) and as adopted by the Laboratory Animal Center, National University of Singapore, were followed. For luciferase activity studies, 4 rats were used for each experimental group. For immunohistochemical study, 2 rats were used per group for sham-operated group, BV-CMV E/PDGF injected group, and BV-CMV injected group.

4.3.6 Brain Injection Methods

For intra-striatum injection, rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (60mg / kg of body weight) and positioned in a stereotaxic instrument with the tooth bar set at 0. Virus particles were separately and bilaterally injected into the striatum at either 3 injection points [coordinates (from bregma and dura): anterior (A), +1.5 mm, lateral (L), +2.0 mm, ventral (V), –5.0 mm; A, +0.5 mm, L, +3 mm, V, -5.0mm and A, -0.3 mm, L, +3 mm, V, -5.0mm] or at 1 point [A, +0.5 mm, L, +3 mm, V, -5.0mm]. For each injection, the injection value varied from 1 μl to 10 μl which contained different titers of viral particles. The speed of injection was 0.5 μl / minute and the needle was allowed to remain *in situ* for 5 minutes before being slowly retracted at the end of each injection (Fig. 4-5).

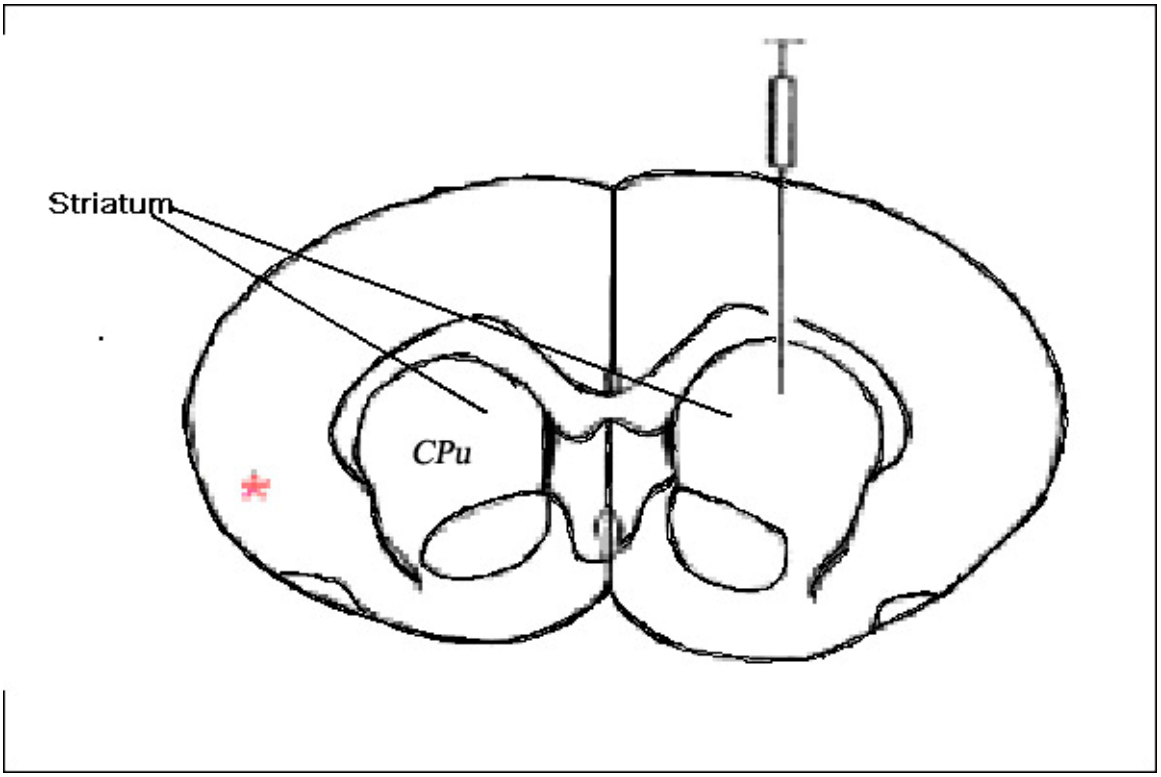


Fig. 4-5. Schematic picture of intrastriatum injection method

4.3.7 Luciferase assay

Cells were collected for luciferase assay after removing the culture medium, and then were freeze-and-thawed for two times in reporter lysis buffer (Promega) to release the luciferase protein from the cytoplasm. Supernatant were collected by centrifugation at 13,000rpm for 5 mins at 4°C. Ten microliters of the supernatant was used for the luciferase activity assay (Promega) at room temperature. Measurements were made in a single-well luminometer (Berthold Lumat LB 9501) for 10 seconds.

For luciferase activity assays for tissue samples, 6 rats per proup were sacrificed by intra-cardiac perfusion with 0.1 M PBS (pH 7.4) following deep anesthesia. The brain was taken out and cerebral cortex, striatum and substantia nigra were dissected and stored

separately at -80°C until further processing. Each sample was homogenized by sonication in PBS buffer (100 µl PBS per 50 mg tissue) for 10 seconds on ice, and then centrifuged at 13, 000 rpm at 4°C. The supernatants were used for luciferase assay as described above.

4.3.8 Immunohistochemical analysis

One day after intrastriatum injection of Cy3 labeled virus and two days for unlabeled baculovirus, rats were sacrificed (n = 2). Following deep anesthesia, all rats were perfused first with 0.1M PBS (pH7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After perfusion, the brains were removed and post-fixed in the same fixative for 2-4 hours before they were transferred into 0.1 M PBS containing 20% sucrose for incubation overnight at 4°C. Frozen coronal sections of each brain were cut at 30µm thickness and stored in 0.1M PBS. Free-floating sections were washed for 20 min in 0.1M PBS at pH 7.4 containing 0.2% Triton X-100, then blocked with 1% blocking solution (diluted 10 folds in 0.1M PBS containing 0.2% Triton X-100, included in Western blotting kit, Roche) for 1 hour. Sections were then incubated overnight with the following primary antibodies: polyclonal antibody against luciferase (Promega; dilution 1:150) and monoclonal antibody against neuron-specific nuclear protein (NeuN) (Chemicon International, USA; dilution 1:500) or monoclonal antibody against glial fibrillary acid protein (GFAP) (Chemicon; dilution 1:500). Then, sections were washed in 0.1 M PBS and further incubated with anti-rabbit IgG conjugated by TRITC(Sigma-Aldrich, Inc., USA; dilution 1:100) and anti-mouse IgG conjugated by FITC (Sigma-Aldrich; dilution 1:100) for 1 hour. After incubation, sections were washed six times in PBS before being spread on gelatin-coated slides. Then, sections were mounted with

fluorescent mounting medium (DAKO) and covered with cover-slips. Control sections were incubated without primary antibodies.

Cy3-labeled virus was used to show the virus internalization in vivo. For this group, sections were incubated overnight only with monoclonal antibody against neuron-specific nuclear protein (NeuN) (Chemicon International, USA; dilution 1:500) or monoclonal antibody against glial fibrillary acidic protein (GFAP) (Chemicon; dilution 1:500). Sections were washed in 0.1 M PBS and further incubated with anti-mouse IgG conjugated by Fitc (Sigma-Aldrich; dilution 1:100) for 1 hour. The sections were then treated as mentioned above.

For in vitro studies, cells were stained in 8-well glass chambers following the same procedure as for tissue samples. After the incubation with secondary antibody, cells were kept in PBS in 4^0C prior to confocal microscopy.

4.3.9 Visualization of double labeling with confocal scanning microscopy

Sections were examined with a Carl Zeiss LSM510 confocal laser scanning microscope. For the detection of FITC fluorescein, each section was initially scanned with a 488 nm laser line, and an emission filter BP 510-525; For the detection of TRITC or Cy3 fluorescein, each section was scanned with a 543 nm laser line, and an emission filter LP 570.

4.4 Results

4.4.1 Visualization of baculovirus entry in vitro and in vivo

The focus of this study was to examine the possibility of infectious entry of baculovirus into neurons. To accomplish this, we have developed methods of conjugating fluorophores, which is Cy3, directly to the baculovirus envelop while preserving the viability and ability of the virus to interact with the cells. labeled virus was used for *in vitro* and *in vivo* gene transfer experiments to check for its gene delivery efficacy.

4.4.1.1 Baculovirus entry into differentiated PC12 cells

Firstly, fluorescently labeled virus particles were used to transfect differentiated PC12 cells. PC12 cells were transfected by baculoviruses at multiplicity of infection (m.o.i) of 5 (about 40 folds lower than the titer used for testing the luciferase expression), and were collected and fixed after 30 minutes, 2 hours and 4 hours exposure to virus, respectively. With Cy3-labeling of baculovirus envelope protein, the internalized viral particles in the cytoplasm were visualized under confocal microscope (Fig.4-6). About half an hour after viral infection, a large number of virus particles were found to be located along the cell membrane. 2 hours after infection, most virus particles dislodged from cell membrane and moved towards the nucleus through the cytosol. 4 hours after infection, significant numbers of virus particles were detected binding along the nuclear membrane.

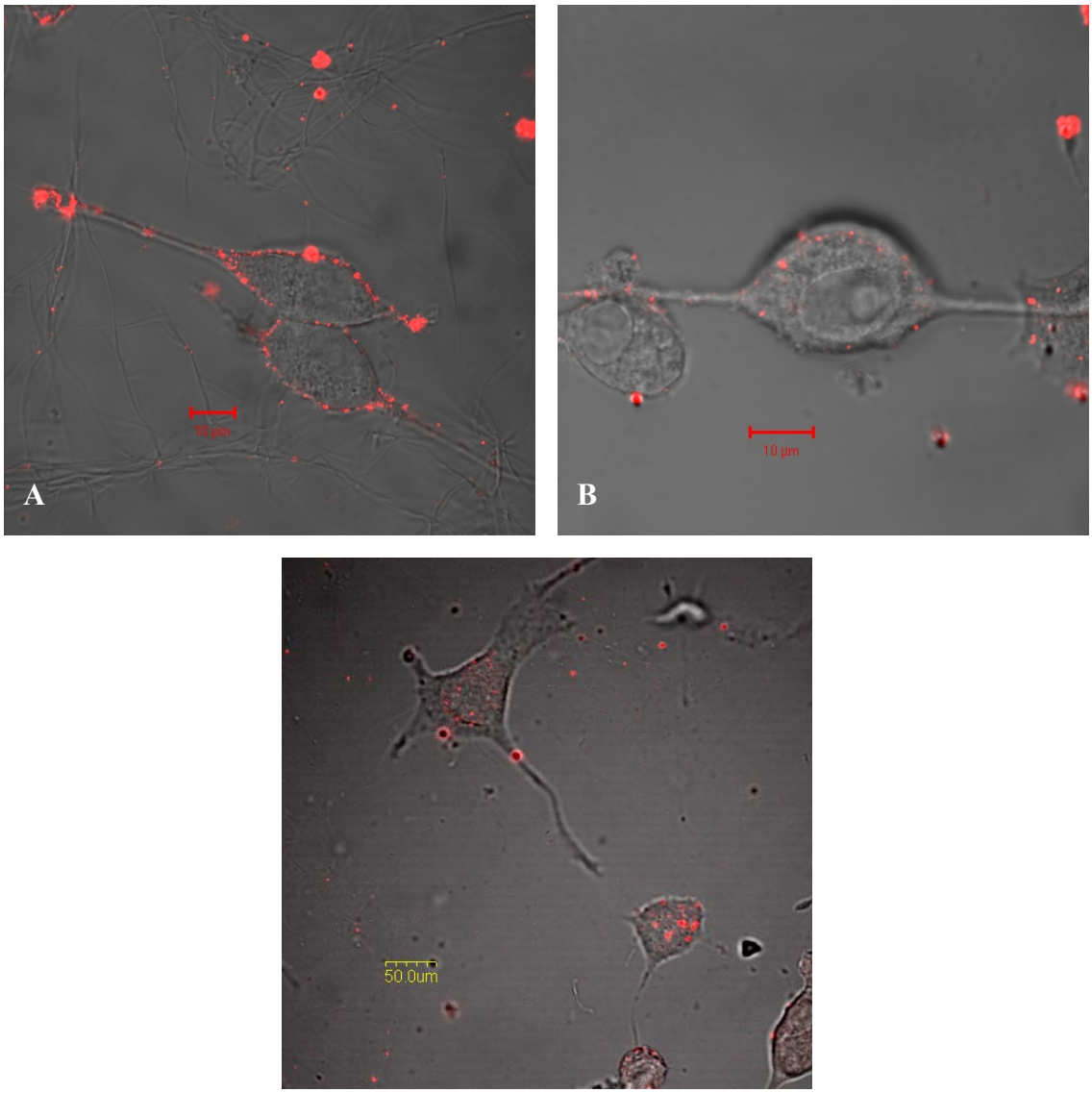


Fig. 4-6. Confocal images of Cy3-labeled baculovirus internalized by differentiated PC12 cells. Cells were collected and fixed at half an hour (A), 2 hours (B) and 4 hours (C) after virus inoculation. Red scale bar represent 10µm; and yellow scale bar for 50 µm.

4.4.1.2 Baculovirus entry in primary neuron cells

To test the infectious ability of baculovirus in terminally differentiated cells, mixed primary neural cell cultures were infected by Cy3-labeled baculovirus vector at m.o.i. of 100. Cells were collected at 4 hours after inoculation, and fixed for immune staining

using antibody against neuronal nuclear protein (NeuN, a neuronal specific marker) to show neurons. Figure 4-7 shows that red signals, representing the virus particles, co-localized in the cytoplasm of primary neurons which is NeuN-positive.

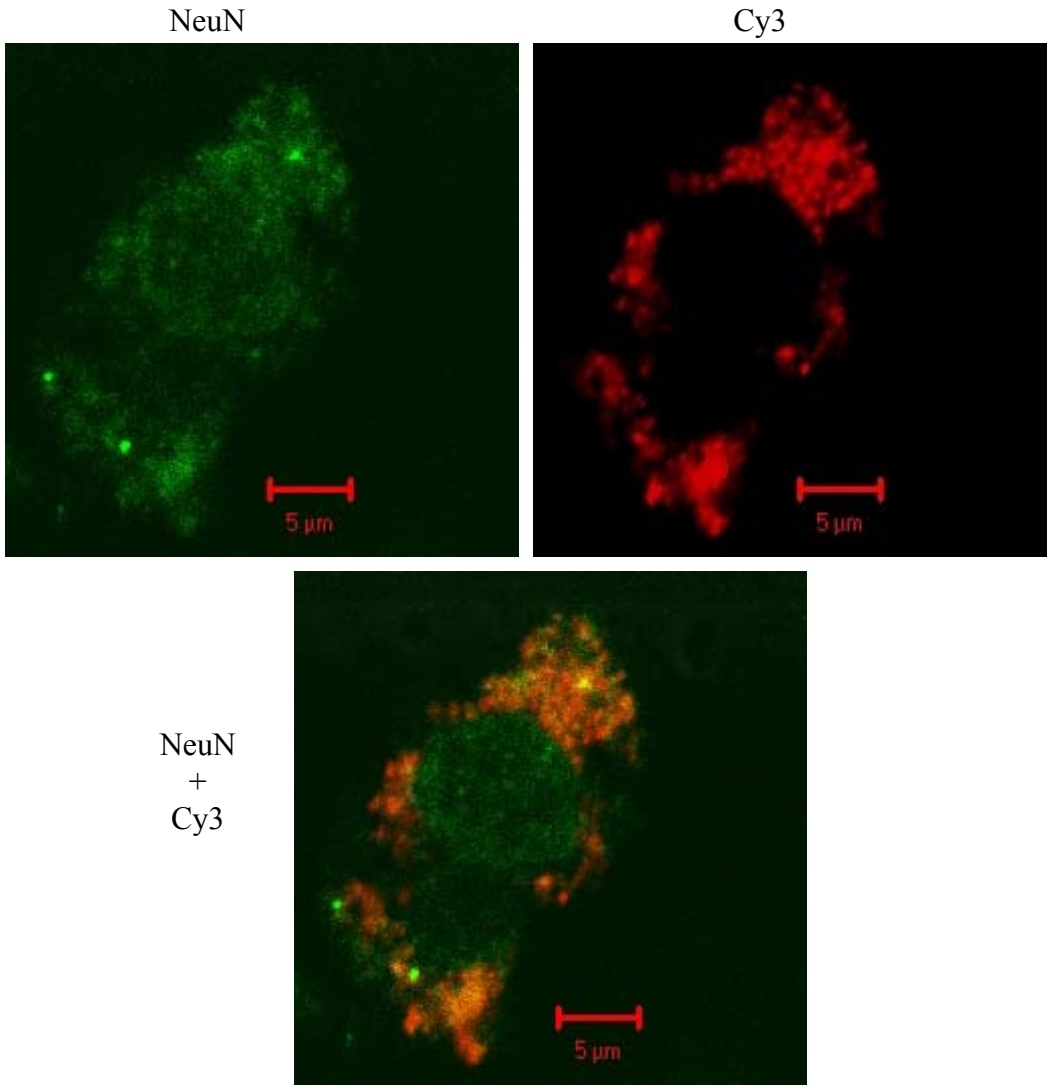
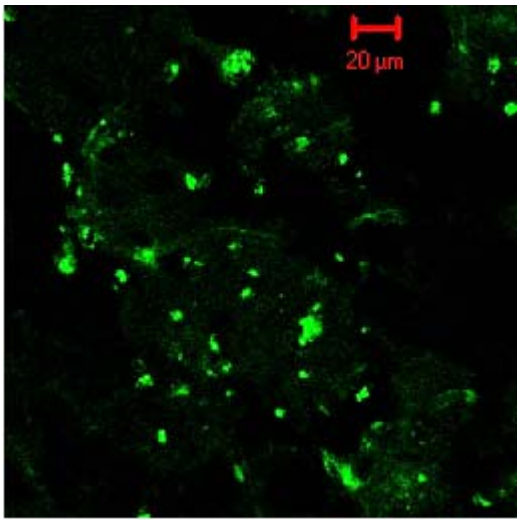
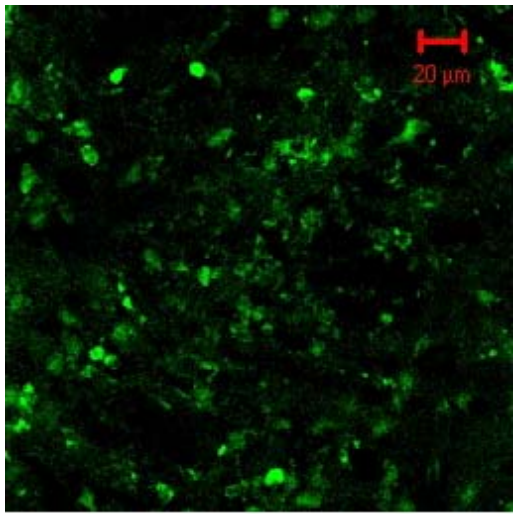


Fig. 4-7. Confocal images of Cy3 labeled baculovirus internalized by primary neurons. Cells were infected by baculovirus vectors at m.o.i. of 100, and were collected and fixed at 4 hours after infection. Scale bar stands for 5µm.

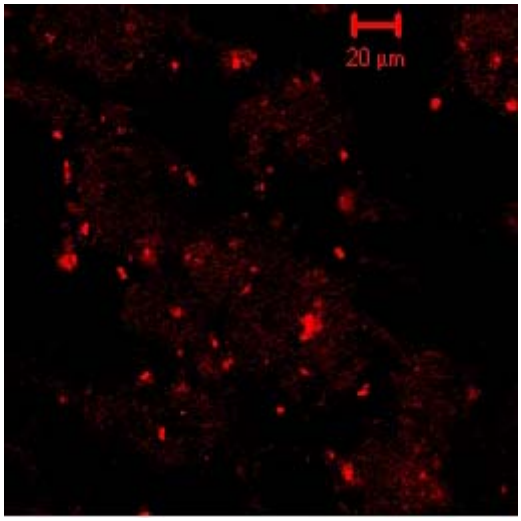
GFAP



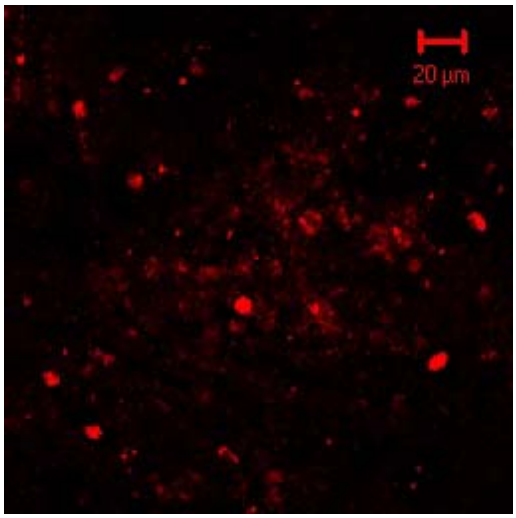
NeuN



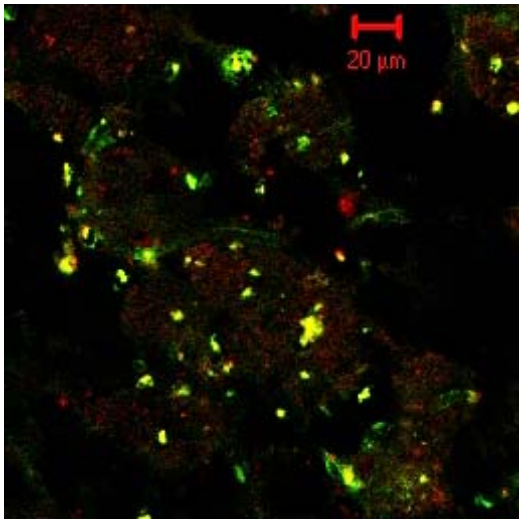
Cy3



Cy3



Cy3+GFAP



Cy3+NeuN

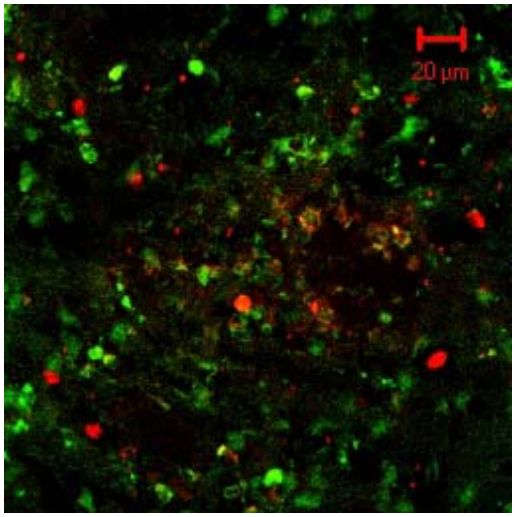


Fig. 4-8. Confocal images of Cy3 labeled virus taken up by neurons and glia cells in rat striatum. Cy3 labeled BV-CMV E/PDGF was injected into the striatum with 10^9 pfu of virus particls as stated in "materials and methods". 1 day after injection, rat brains were collected. Frozen coronal sections of each brain were cut at 30μm thickness and used for immune staining against neuron-specific nuclear protein (NeuN) to show neurons, or against glial fibrillary acidic proteins (GFAP) to show glial cells.

4.4.1.3 Baculovirus entry in neural cells in vivo

Cy3 labeled BV-CMV E/PDGF was injected into rat striatum by a stereotaxic apparatus to investigate the cell tropism of the baculovirus in neural cells of CNS. Brain tissues close to the injection site were collected and sectioned 1 day after virus infection. The cryostat sections were then stained by anti-NeuN and anti-GFAP. Figure 4-8 shows the confocal images of sections of the striatum. The Cy3 signals were co-localized with both NeuN and GFAP-positive marker, demonstrating the internalization of virus particles into both neurons and glia cells. The big bright red signals were supposed to be the virus clump, which cannot be taken up by neurons but may be engulfed by microglia cells. The ratio of the transfected neurons to glia cells is about 3:7, by quantitative analysis. BV-CMV vector displays the same pattern in virus entry into neurons and glia cells.

4.4.2 Neuron-specific gene expression derived from BV-CMV E/PDGF in vitro and in vivo

4.4.2.1 Neuron-specific gene expression in primary neural cells

The neuron-specific transgene expression of the BV-CMV E/PDGF was tested firstly in the mixed primary neural cell cultures obtained from embryonic rat brain, using the BV-CMV infected group as control. Two days after infection, cells were double stained with anti-luciferase and anti-NeuN or anti-GFAP. As shown in figure 4-9, A-F, most of the luciferase-positive cells are also NeuN-positive cells (Fig. 4-9C) from BV-CMV E/PDGF infected group, with only a few luciferase positive cells being GFAP-positive cells (Fig. 4-9F). However, BV-CMV drived luciferase expression are found more in GFAP-positive cells (Fig. 4-9L) than in the NeuN-positive cells (Fig. 4-9I). This finding demonstrates that the hybrid neuronal specific promoter, CMV E/PDGF, plays a key role in achieving neuronal specific gene expression for baculovirus vector.

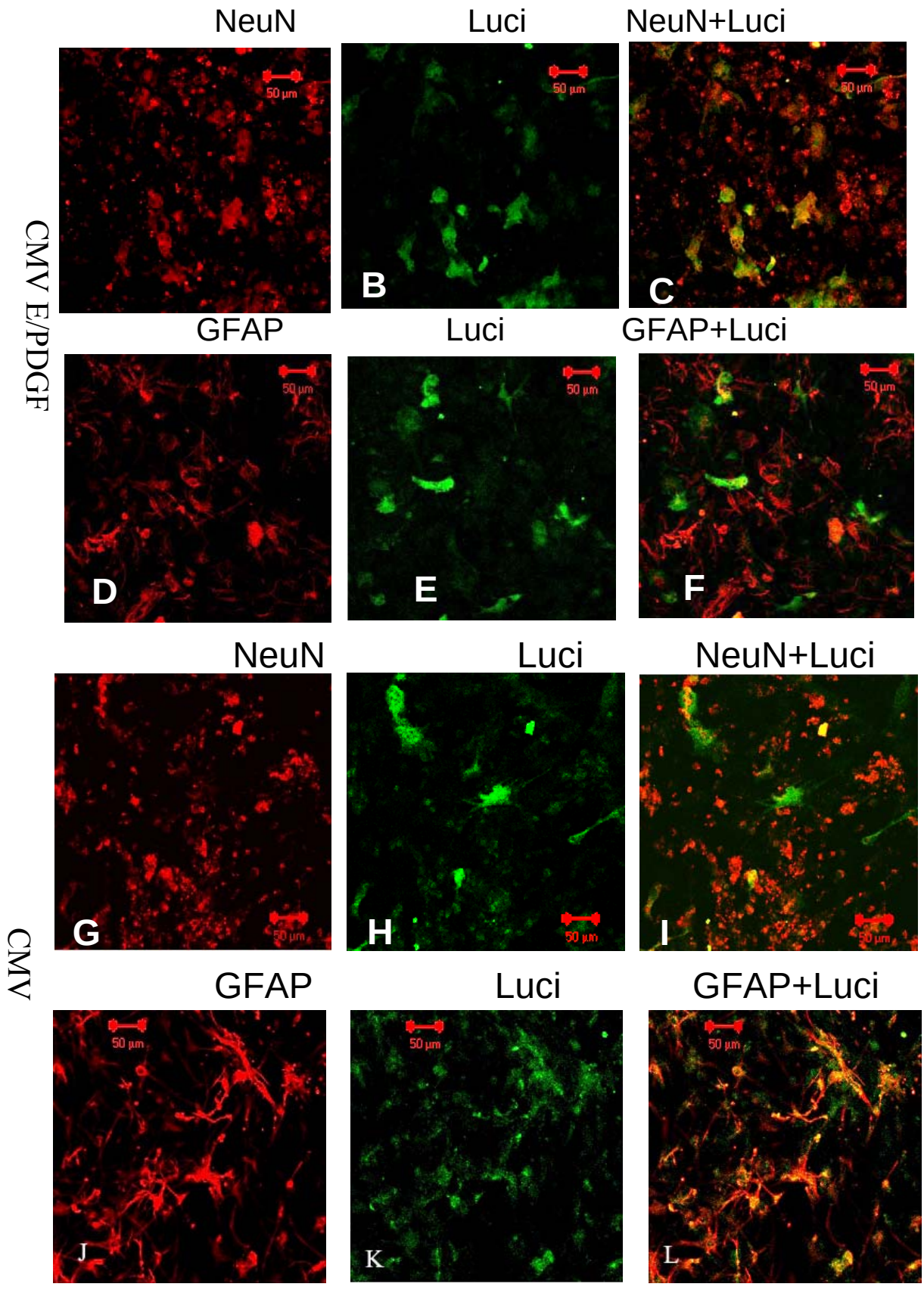

NeuN
Luci
NeuN+Luci
CMV E/PDGF
B
C
GFAP
Luci
GFAP+Luci
D
E
F
NeuN
Luci
NeuN+Luci
CMV
G
H
I
GFAP
Luci
GFAP+Luci
J
K
L

Fig. 4-9. Confocal images of luciferase expression in mixed primary neural culture with double-staining. Virus at a m.o.i. of 200 was used for inoculation. Green signals show transfected cells with luciferase expression; red signals show neurons or glial cells. A-F were BV-CMV E/PDGF infected group; G-L were BV-CMV infected group. Scale bar stands for 50μm for A-I, and 20 μm for J-L.

4.4.2.2 Neuron-specific expression in brain after intrastriatum injection

We next investigated the BV-CMV E/PDGF mediated transgene expression in the rat striatum with BV-CMV included for comparison. Immunohistochemical analysis was carried out two days after stereotaxic injection of 5×10^6 pfu of virus particles into the rat striatum. Cryostat sections of striatum around the injection site were cut at 30μm thickness and were double stained with anti-luciferase and anti-NeuN (Fig. 4-10). In the BV-CMV E/PDGF infected striatum, most of the cells expressing luciferase were positively stained by antibody against NeuN (Fig. 4-10A-C), demonstrating that the transgene expression was confined to the neurons. By contrast, in the BV-CMV infected group, only a small percentage of luciferase-positive signals were localized in the NeuN-positive cells, which is consistent with the previous reports that baculovirus vectors bearing viral promoters such as CMV, CAG, demonstrated very poor neuronal expression capability in the brain (Lehtolainen et al., 2002a;Sarkis et al., 2000).

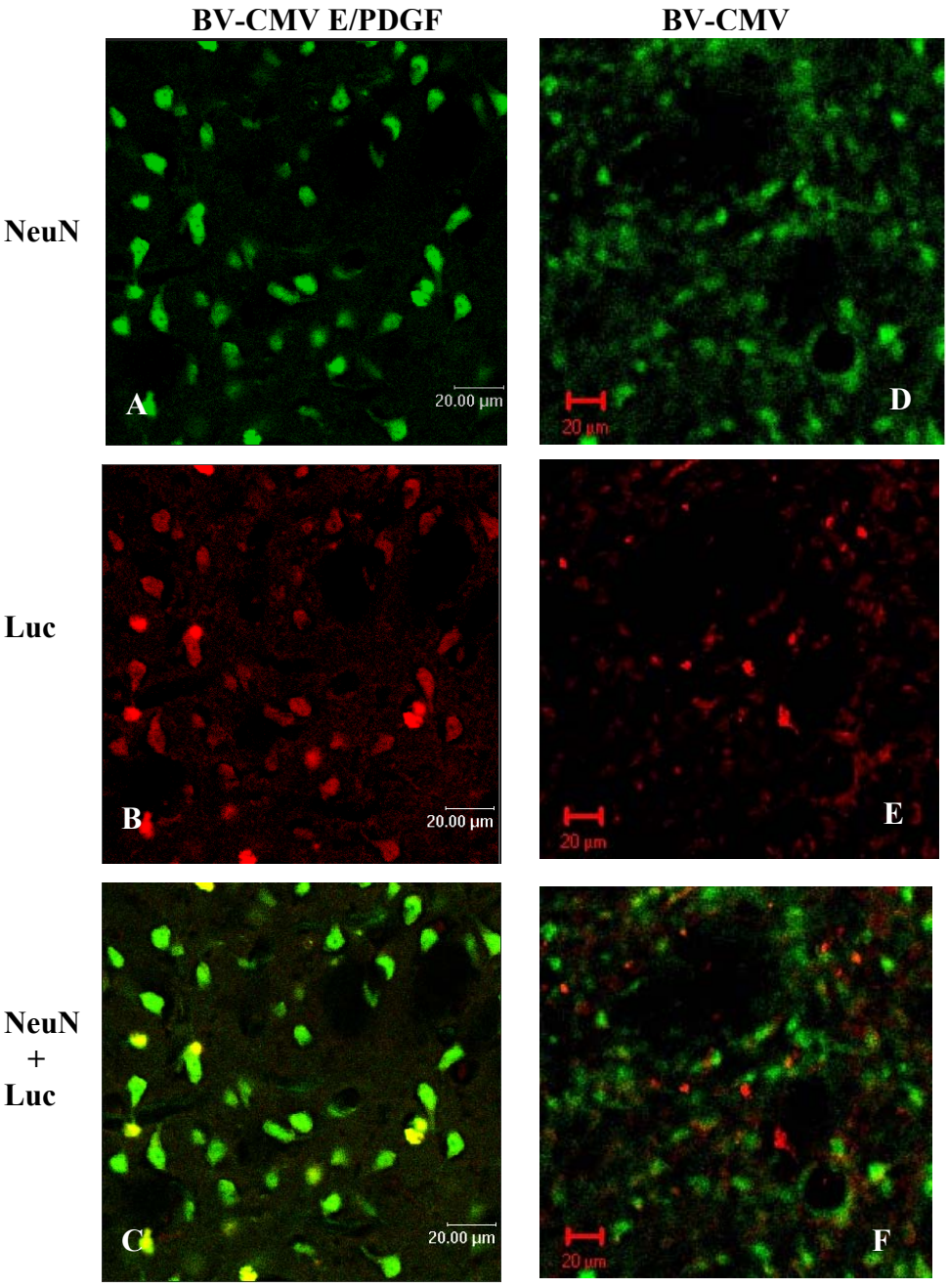


Fig. 4-10. Confocal images of luciferase expression in NeuN-positive cells in the rat striatum. A-C were BV-CMV E/PDGF infected group, and D-F were BV-CMV infected group. Red signal stands for luciferse expression; green signal stands for NeuN-positive cells. Scale bars stand for 20µm.

4.4.3 Prolonged transgene expression derived from BV-CMV E/PDGF in vitro and in vivo

4.4.3.1 Prolonged transgene expression in primary neural cell cultures

An in vitro study was carried out in primary neurons and primary glia cells using BV-CMV E/PDGF, BV-PDGF and BV-CMV to examine the long-term transgene expression profile of the respective viral vectors. Cells were infected by purified and concentrated virus at a m.o.i. of 200. As shown in figure 4-11, BV-CMV E/PDGF showed significantly improved transgene expression to about 77% of that derived from BV-CMV, and 10 folds higher than that from BV-PDGF in primary neuronal cultures. After 6 days, the expression level from BV-CMV E/PDGF group remained unchanged, while that of the other two groups dropped drastically to a nearly undetectable level. On the other hand, in primary glia cell cultures, no increment of transgene expression could be detected from BV-CMV E/PDGF when compared with the BV-PDGF treated group. And 6 days after infection, all the three groups showed no detectable transgene expression. This finding further proved that the improved and prolonged transgene expression derived from BV-CMV E/PDGF is confined to neurons.

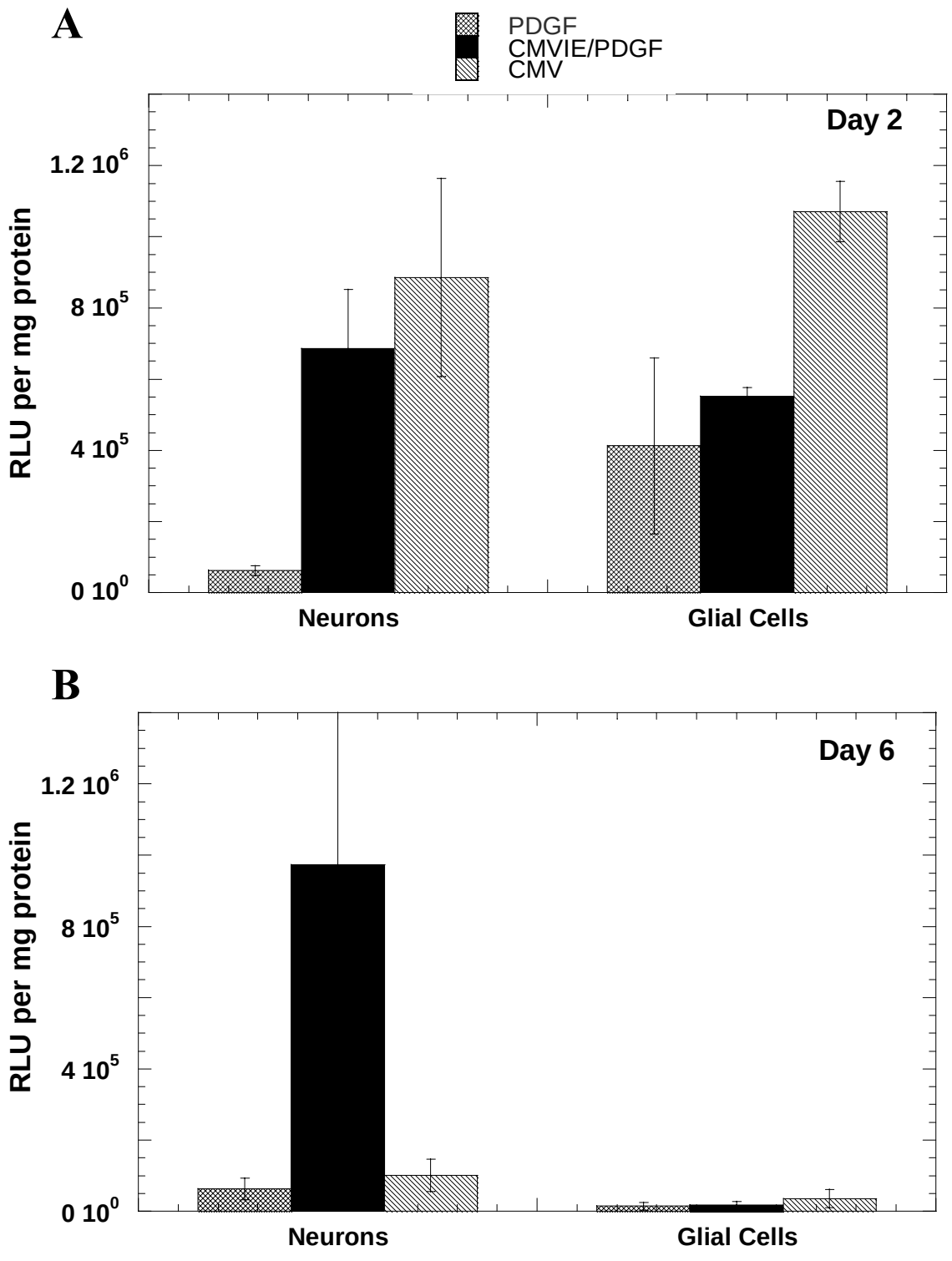


Fig. 4-11. Activities of three different baculovirus vectors in primary neural cultures. A&B cells collected 2 or 6 days after infection, respectively. Results are expressed as RLU/mg protein ± SD.

4.4.3.2 Dose-response study in rat brain after intrastriatum injection

A dose-response study was then carried out for BV-CMV E/PDGF in rat striatum, with BV-CMV and BV-PDGF as comparisons (Fig. 4-12). Rats were infected with $1x10^6$, $5x10^6$, or $1x10^7$ p.f.u. of viral particles, respectively. Two days after infection, three parts of brain tissues, striatum, cerebral cortex and substantia nigra, were collected for luciferase activity assay. The value of each part of brain was added up and presented as RLU/brain (relative luciferase unit per brain). Luciferase expression increased along with the increment of virus titer from both BV-CMV E/PDGF and BV-CMV infected groups. However, BV-PDGF mediated very low luciferase expression, and no increment of luciferase activity could be found with the increase in virus titer. In vitro study carried out on PC12 cells, using BV-CMV or BV-CMV E/PDGF, also exhibited an increasing, linear dependence of the luciferase expression on the viral titer up to 1200 m.o.i (data not shown).

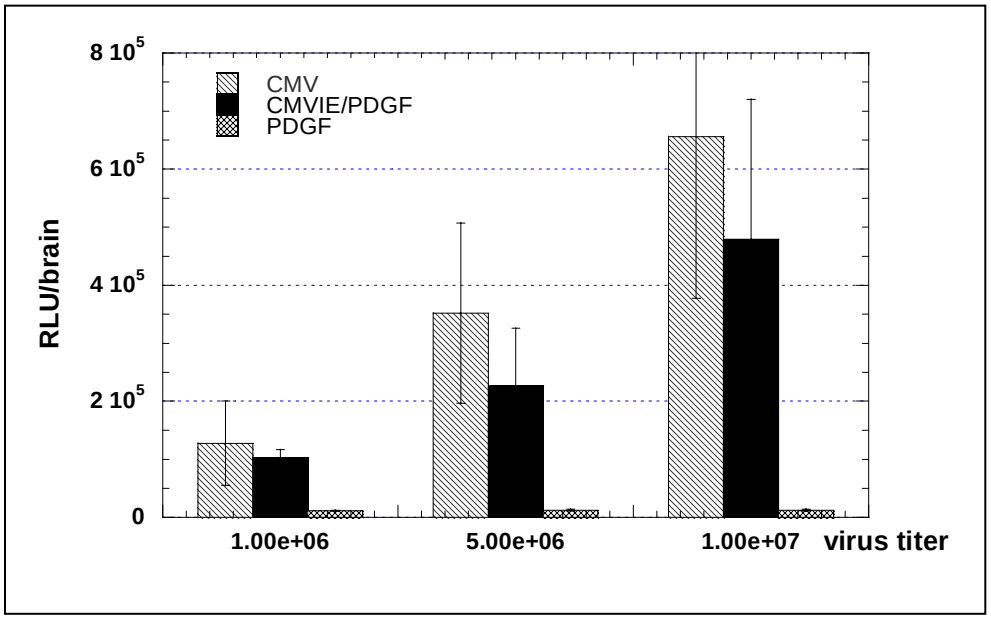


Fig. 4-12. Dose respondence of baculovirus infection after injection into rat striatum. Four rats were used per time point. Values are presented as means ± SEM.

4.4.3.2 Time course study in rat brain after intrastriatum injection

An in vivo time-course study was also carried out using BV-CMV E/PDGF and BV-CMV to investigate the kinetics of gene expression from baculovirus accommodating different promoter (Fig. 4-13). $1x10^7$ p.f.u of purified and concentrated baculovrius particles were injected into the rat striatum. At 1 day after infection, BV-CMV E/PDGF mediated luciferase expression was only about 20% of that derived from BV-CMV. The expression level from BV-CMV E/PDGF peaked at 3 days after infection, while that from BV-CMV decreased to about 70% of the expression from BV-CMV E/PDGF. At two weeks after infection, when BV-CMV mediated expression dropped to about $8x10^4$ RLU/brain, the luciferase expression remained at about $6.4x10^5$ RLU/brain from the BV-CMV E/PDGF infected group. After 1 month, luciferase expression remained constant for the BV-CMV E/PDGF group, which is about 9.5-fold higher than that of BV-CMV treated group at that time point.

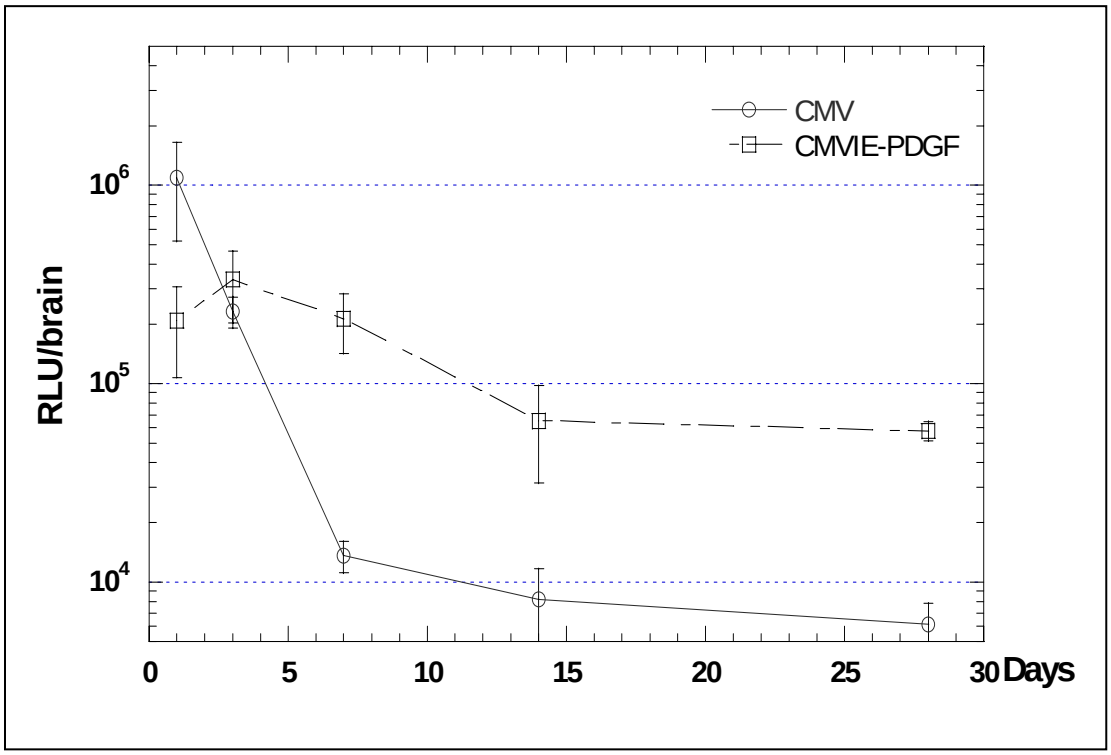


Fig. 4-13. Time course of luciferase expression after baculovirus injection into rat striatum. Brain tissues were collected for luciferase assay at time points of 1, 3, 7, 14 and 28 days after virus infection, respectively. Four rats were used per time point. Values are presented as means ± SEM.

4.5 Discussion

What attributes should an ideal viral vector have for gene therapy of neurodegenerative diseases? Encompassed within these requirements are a number of features, including neuron-preferential transfection, persistent transgene expression and ideally, a large cloning capacity and relative easy ways to produce high titers. Most importantly, the vector must be safe, nontoxic, and not inhibited by a preexisting humoral or cellular immunity. With these in mind, we considered using recombinant baculovirus vector, which has large insert capacity and is relatively safer than other commonly used viral vector such as adenovirus, HSV, etc (Hartig et al., 1992;Doller et al., 1983). In this report,

a new baculovirus vector, BV-CMV E/PDGF, was constructed to mediate neuron-specific and prolonged transgene expression by using a hybrid neuron-specific promoter.

Baculovirus was reported to show hepatocyte tropism at the early stages of these being used as a mammalian gene delivery vector (Boyce and Bucher 1996;Hofmann et al., 1995;Ni 2001b). But, a broad spectrum of cell types were found to be successfully transfected by this vector later (Ghosh et al., 2002), although with different transfection efficiency, suggesting that this viral vector have a wide cell type tropism. The mechanism of the primary binding step of baculovirus to mammalian cell membrane has been studied by different groups, but their results are not consistent. Dose dependent saturable kinetics was exhibited in transfection of Huh7 cell (Hofmann et al., 1995), while unsaturable viral entry was reported in the transfection of 293 cells (Duisit et al., 1999). In this study, we also observed unsaturable transfection of PC12 cells by recombinant baculovirus vector (data not shown). Competitive entry into cells between reporter gene expression vector and non-expression vector was also detected in the first study (Hofmann et al., 1995), but not in the later one (Duisit et al., 1999). One possible explanation for these conflicting data is that the mechanism for virus–cell interactions might be different for different cell types. Depletion of major negative charged group on the cell surface, such as phosphatidic acid, phosphatidylinositol (Tani et al., 2001), or masking the cell membrane with positive charged molecules, like heparin and polybrene (Duisit et al., 1999) will significantly diminish the transfection efficiency of recombinant baculovirus. Moreover, this virus can also bind to heparin columns in vitro. These evidences listed above indicate that electrostatic charge interaction between the viral envelope and host cell membrane is

the initiating step of virus binding, which is likely to be a nonspecific process. Several kind of neural cells, such as glial cells, were successfully transfected by baculovirus vector in vitro and in vivo (Lehtolainen et al., 2002b;Sarkis et al., 2000), with no direct evidence of neuronal transfection, except for an unpublished data mentioned in Sarkis's study that neurons can be transfected by baculovirus incorporating a Rous sarcoma virus (RSV) promoter (Sarkis et al., 2000).

In this study, a fluorescently labeled virus was used to follow the virus internalization into neurons. The results demonstrate that a baculovirus vector, no matter what promoter it carries, can enter primary neurons or neurons in the striatum efficiently. The number of transfected glia cells in striatum is more than that of neurons, which may be explained by the number of glia cells in CNS being overwhelmingly more. Thus, the restricted transgene expression in choroid plexus endothelial cells previously reported by Lehtolainen et al(Lehtolainen et al., 2002b), after virus delivery into the corpus callosum, may be due to the lack of neurons at the injection site, corpus callosum, which is abound with nerve fibers and glial cells. The other concern could be that a non-specific viral promoter, CMV, may not specifically facilitate transgene expression in neurons.

Utilizing neuronal specific promoter for targeted gene expression in neurons is a very efficient and commonly used strategy in viral gene delivery to the CNS**.** Neuron-targeted gene expression has been achieved for adenovirus(Morelli et al., 1999), AAV(Fitzsimons et al., 2002), lentivirus (Jakobsson et al., 2003), and HSV(Jin et al., 1996b). This study is the first one to report a baculovirus vector with neuron-targeted transgene expression

using a neuronal specific promoter. After incorporating the hybrid promoter into the context of the baculovirus vector, BV-CMV E/PDGF demonstrates high neuronal specificity in both primary neural cell cultures and rat striatum. While in primary neural cell cultures, some expression in glial cells was detected, the expression in striatum is restricted to neurons. This phenomenon may be explained by a change in the phenotype of some of the primary cells during culture processing. The disruption of the intercellular junctions for example may influence the cells by some unknown mechanism, which facilitated the baculovirus derived gene expression in glial(Bilello et al., 2001). Other explanation may be that the primary glia culture was contaminated by a few neuronsl.

Prolonged transgene expression is always preferred in gene therapy for neurodegenerative diseases, characterized by long term chronic process. Baculovirus vector bearing a viral promoter such as CMV and CAG, however, gives high transient transgene expression both in vivo and in vitro, but drops rapidly by orders of magnitude, as observed in the current study and reported by the previous study (Shoji et al., 1997). Promoter inactivation rather than loss of vector DNA has been suggested to be the primary factor limiting long-term gene expression (Gill et al., 2001;Yew et al., 2001). Possible causes include hypermethylation of promoter and depletion of endogenous activating transcription factors (Loser et al., 1998; Prosch et al., 1996b). Cellular promoters are usually less sensitive to inactivation than viral promoters, as already been observed with adenoviral and retroviral vectors (Navarro et al., 1999; Palmer et al., 1991). It is not clear that if the rapid decline of BV-CMV mediated transgene expression is due to the inactivation of CMV promoter. CMV promoter is known to be susceptible to

transcriptional inactivation by the methylation of cytosines in CpG dinucleotide (Prosch et al., 1996a; Jin et al., 1996a), although it remains unaffected in lentivirus vectors (Naldini et al., 1996). The fewer CpGs contributed by CMV enhancer and the cellular authentic sequences in the PDGF may be the crucial parameters defining the persistence of transgene expression from BV-CMV E/PDGF.

In conclusion, the novel recombinant baculovirus vector, BV-CMV E/PDGF, displayed high neuron-specificity and sustained transgene expression both in primary neural cultures and in rat brain. These attributes, together with its biosafety, large insertion capacity, etc, establish this new vector a very promising gene carrier for CNS gene therapy.

CHAPTER 5 AXONAL TRANSPORT OF RECOMBINANT BACULOVIRUS VECTOR

5.1 Abstract

Targeted gene delivery to neurons is crucial to effective gene therapy of neurodegenerative diseases. Several types of viral vectors can target neurons through retrograde axonal transport to somas of projection neurons after viral internalization at axon terminal fields. We demonstrated for the first time that recombinant baculovirus vectors could migrate by axonal transport, resulting in transgene expression in projection neurons. After stereotaxic aided injection of Cy3-labled baculovirus vectors into the rat striatum, retrograde axonal transport of these vectors was observed along the corticostriatal pathway and nigrostriatal pathway. After intravitreous body injection, anterograde axonal transport and trans-synaptic transport of the virus particles were observed in defined connections of the visual system, from the retina and the optic nerve to the lateral geniculate body, the superior colliculus and the primary visual cortex. PCR analysis confirmed the existence of transported viral DNA in the tissue samples collected from projection fields. Transgene expression was also detected in the target regions remote to an injection site by luciferase assay and immunohistological analyses. The attributes of baculovirus vectors in the bidirectional and transneuronal transport in neural circuits would benefit targeted gene delivery in CNS.

5.2 Introduction

The obstacles to efficient gene delivery into neurons affected in neurological disorders include the broad anatomical distribution of some of the neurons and the relatively inaccessible location of some others, making direct local delivery of therapeutic agents problematic. Several viral vectors seem to be capable of overriding this problem by taking advantage of the natural process of axonal transport. This process begins with endocytosis of extracellular substances. In the case of axonal endocytosis, axon terminals are used as ports of entry to take up these substances. The incorporated substances are then transport along the axon to the cell body. The viruses capable of doing so include adenovirus (Peltekian et al., 2002a; Choi-Lundberg et al., 1998; Hermens et al., 1997; Bilang-Bleuel et al., 1997b; Soudais et al., 2001a), adeno-associated viruses (AAV) (Kaspar et al., 2002d) and herpes simplex virus (HSV) (Breakefield and DeLuca 1991;Jin, et al., 1996). Compared to baculovirus, adenovirus and HSV elicit much stronger immune and inflammatory responses. A single intra-cerebral injection of either adenovirus or HSV results in dose-dependent inflammatory reactions in the brain leading to demyelination (Lawrence et al., 1999;McMenamin et al., 1998). AAV vectors, which are able to integrate into the target cell chromosome, are characterized by the inability to consistently induce immune responses. Their application as gene delivery vectors is however hampered somehow by their small insert size of the viral vectors, accommodating foreign DNA only up to 4.5 kb, and difficulty in their production (Jooss and Chirmule 2003).

We investigated in the current study whether baculovirus vectors could be transported in axons, thus mediating gene expression in regions remote to the injection site. Intra-striatum injection was used to study retrograde transport in the corticostriatal and nigrostriatal pathway, and intra-vitreous body injection was used to study possible anterograde and trans-neuronal transport in the visual pathway. The recombinant baculovirus vectors accommodating the neuron-specific CMV E/PDGF promoter was used to facilitate targeted gene expression in neuronal cell bodies.

5.3 Materials and methods

5.3.1 Intravitreous body injection

Animals were anaesthetized the same way as the previously discribed.(4.3.6) A syringe with a needle of No.30 (Hamilton) was inserted through the sclera into the vitreous body posterior to the ora seratta, and then 10ul of the vitreous body was slowly aspitated. After that, 10μl of the virus particles (10^7 pfu) was slowly injected back into the posterior chamber.

5.3.2 PCR detection of virus genome in tissue samples

Following deep anesthesia, virus infected rats were sacrificed 2 days after infection by intra-cardiac perfusion with 0.1 M PBS, pH 7.4. Tissue samples were collected and homogenized by mincing with a pestle and the virus genome was extracted according to the standard manufacturer's protocol of a DNeasy Tissue Kit (Qiagen). The primers

targeted a sequence of around 400 base pairs, overlapping the native virus genome and the inserted reporter gene. PCR used primers are listed below:

5′ primer: 5'-AT TGC TCA ACA GTA TGG GCA-3'

3′ primer: 5'-CGA AGA AGG AGA ATA GGG TTG-3'

Amplification cycles consisted of a 7 min denaturation step at 94 °C, followed by 36cycles of 94 °C, 45 s denature, 52 °C 30 s annealing, 72 °C, 30 s extension, 36 cycles and one final extension cycle at 72 °C for 7 min. To exclude false positive resulting from possible contamination during the PCR process, a control rat brain without virus infection was examined in parallel.

5.3.3 Visualization of double labeling with confocal scanning microscopy

Sections were examined with a Carl Zeiss LSM510 confocal laser scanning microscope. For the detection of FITC fluorescein, each section was initially scanned with a 488 nm laser line, and an emission filter BP 510-525; For the detection of TRITC or Cy3 fluorescein, each section was scanned with a 543 nm laser line, and an emission filter LP 570.

5.3.4 Luciferase assay

For luciferase activity assays for tissue samples, 6 rats per proup were sacrificed by intra-cardiac perfusion with 0.1 M PBS (pH 7.4) following deep anesthesia. The brain was taken out and cerebral cortex, striatum and substantia nigra were dissected and stored separately at -80°C until further processing. Each sample was homogenized by sonication in PBS buffer (100 μl PBS per 50 mg tissue) for 10 seconds on ice, and then centrifuged at 13, 000 rpm at 4°C. The supernatants were used for luciferase assay as described above.

Supernatant were collected by centrifugation at 13,000rpm for 5 mins at 4°C. Ten microliters of the supernatant was used for the luciferase activity assay (Promega) at room temperature. Measurements were made in a single-well luminometer (Berthold Lumat LB 9501) for 10 seconds.

5.4 Results

5.4.1 Retrograde transport of virus particle after intra-striatum injection

5.4.1.1 Retrograde transport of Cy3-labeled virus

We first used Cy3 labeled baculovirus vectors to examine the possible retrograde axonal transport of the vectors. $1x10^9$ pfu Cy3 labeled vectors were injected into the Wista rat striatum aided by a stereotaxic aparatus. Tissue sections along the corticostriatal pathway and the nigrostriatal pathway were collected and stained using antibodies against NeuN to show the neurons in striatum and cerebral cortex or antibodies against tyrosine hydroxylase to show dopaminergic neurons in substantia nigra. As Cy3 was conjugated to the envelope proteins of the virus, the fluorescence displayed in the tissue would represent viral particles transported with an intact viral envelope. Figure 5-1 shows the confocal images of sections of striatum, cerebral cortex and substantia nigra. In the striatum, the Cy3 signals were detected in both NeuN-positive and NeuN-negative cells, indicating viral entry in both neuronal and non-neuronal cells. In cerebral cortex and substantia nigra, Cy3 signals were strictly co-localized with either NeuN or TH signals, suggesting that the virus particles entered axon terminals and moved in axoplasm to the somas of projection neurons located in the cerebral cortex and the substantia nigra.

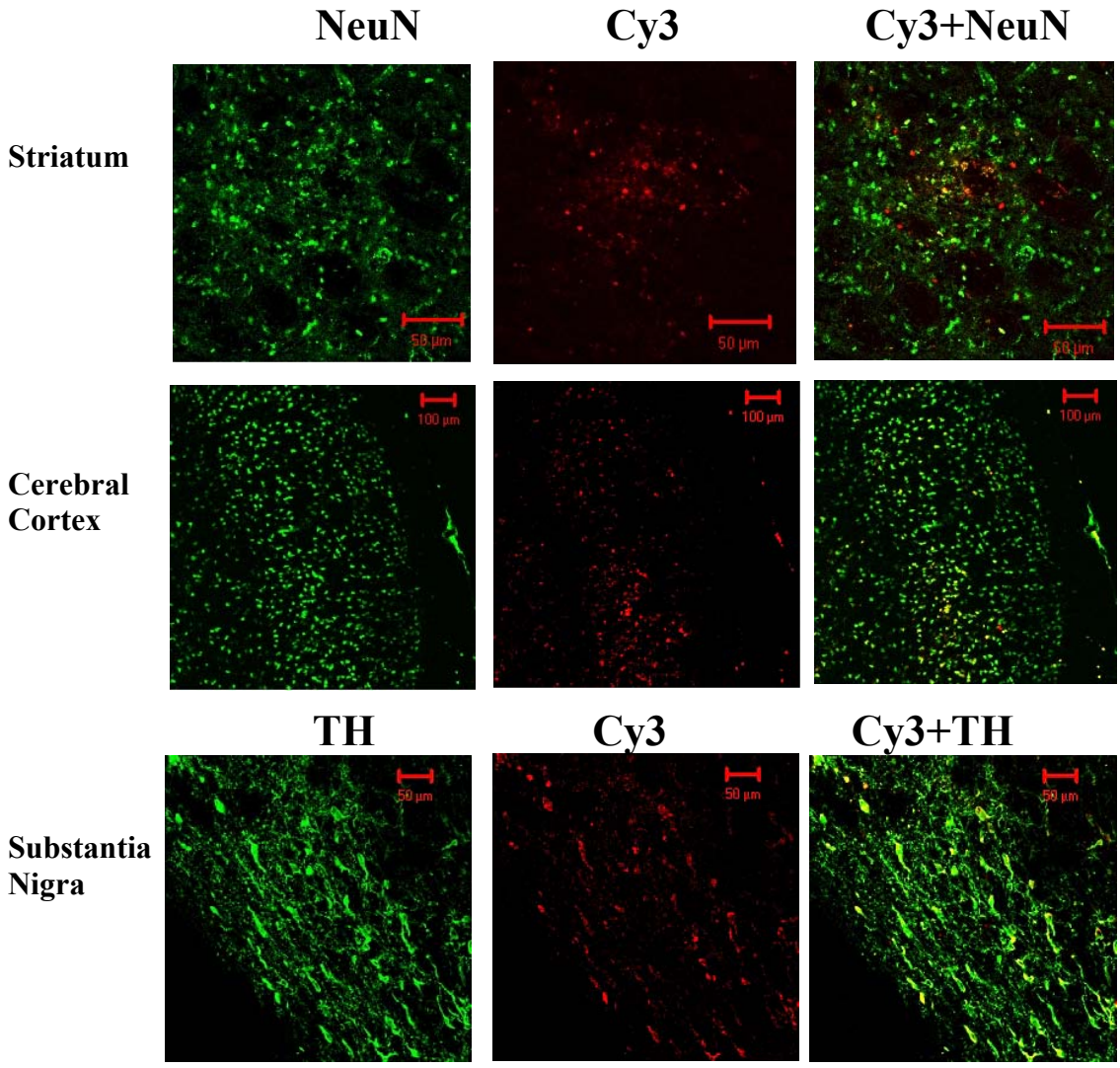


Fig. 5-1.Confocal images of uptake and transport of Cy3 labeled baculovirus in striatal pathway. Green signal stands for atibody against neuron-specific nuclear protein (NeuN) to show neurons in the striatum and cerebral cortex or against tyrosine hydroxylase (TH) to show dopaminergic neurons in the substantia nigra. Red signal stands for Cy3 labeled virus particles. Scale bar present for 50µm for images of striatum and substantia nigra, 100µm for cerebral cortex.

5.4.1.2 Baculovirus DNA detected by PCR analysis

Baculovirus DNA was detected in the cerebral cortex and the substantia nigra remote from the injected striatum by PCR analysis (Fig. 5-2).

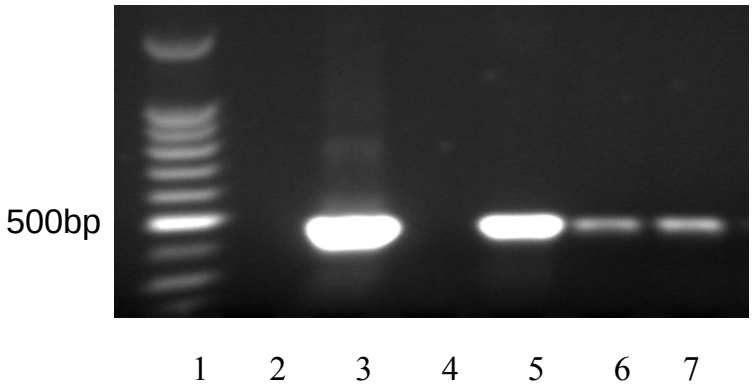


Fig. 5-2 Baculovirus DNA detected by PCR in rat brain after intra-striatum injection. Lane 1, 100bp ladder; lane 2 & lane 4, no injection and shamed injection with 1XPBS as negative control; lane 3, purified BV-CMV E/PDGF genome as positive control; lanes 5, 6 and 7, cerebral cortex, substantia nigra and striatum.

5.4.1.3 Reporter gene expression tested by luciferase assay

To test the possibility of gene transfer in projection neurons, luciferase expression driven by the neuron-specific CMV E/PDGF promoter was examined. Luciferase activity was measured 2 days after injection of $5x10^6$ plaque-forming units (pfu) of recombinant baculovirus. Luciferase activity was detected not only in the injection site but also in distal substantia nigra and cerebral cortex regions (Fig. 5-3). The cerebral cortex contributed more than 90% of the total luciferase activity, while about 1% was presented in the substantia nigra.

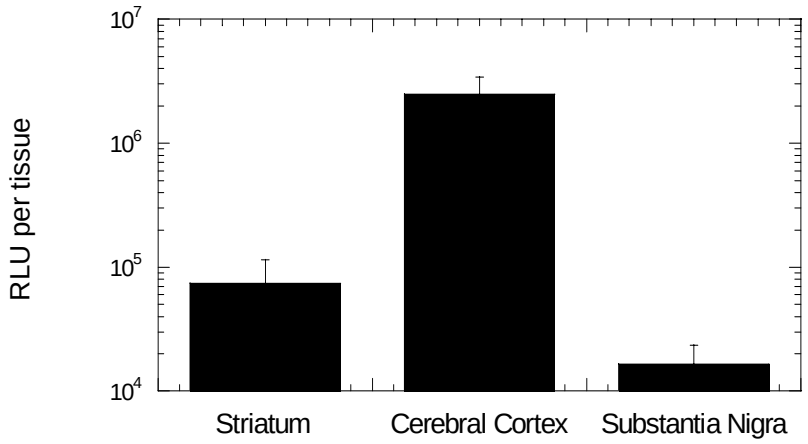


Fig. 5-3. Luciferase gene expression in different brain area after intrastriatum injection. Four rats were used for the experiment. Values are presented as means ± SD.

5.4.1.4 Reporter gene expression localized by double staining

Further experiment were performed to determine the transfected cell type using immunohistological staining. As shown in Figure 5-4, the expression of luciferase reporter gene strictly co-localized with either NeuN-positive neurons in striatum and cerebral cortex or TH-positive neurons in the substantia nigra. In the cerebral cortex, luciferase signals were detected mainly in large pyramidal neurons located in deeper layers, a group of cells known to project their long axons to the putamen of the striatum. There were very few transduced cells, with granule interneuron morphology, in surface layers of the cerebral cortex (Fig. 5-4M-O).

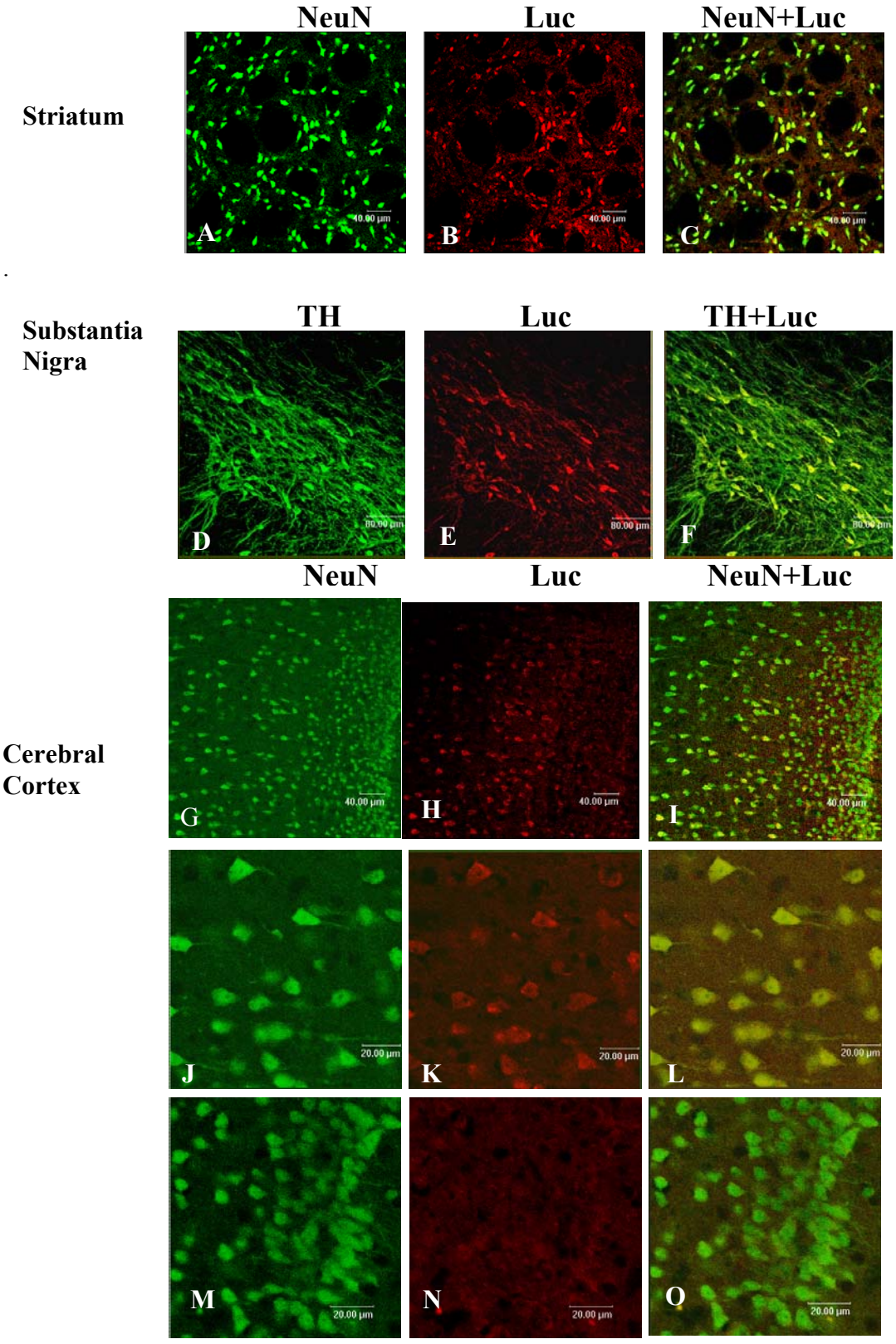


Fig. 5-4.Confocal image showing luciferase expression in neuron after intrastriatum injection. D-F, double staining against luciferase to show transfected cells, against

tyrosine hydroxylase to show dopaminergic neurons in substantia nibra. A-C & G-O, double staining against luciferase and against neuron-specific nuclear protein (NeuN) to show transfected neurons in striatum and cerebral cortex. G-I, low magnification image for cerebral cortex; J-L, high magnification image showing transfected pyramidal neurons. M-O, high magnification showing untransfected granule cells. Scale bar present 80um for D-F, 40um for A-C & G-I, 20um for J-O.

5.4.2 Axonal and anterograde transport of virus particle after intra-vitreous body injection

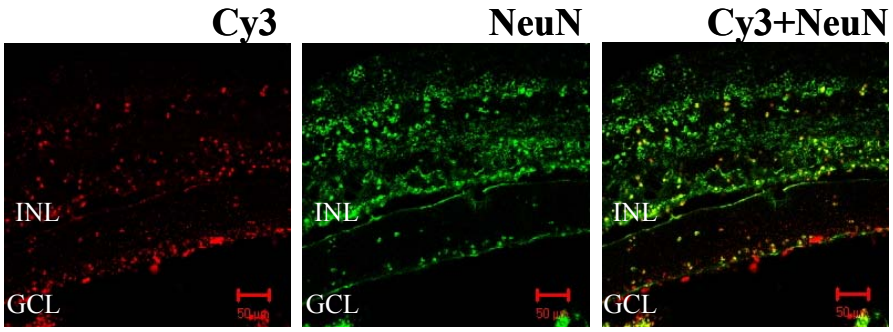
5.4.2.1 Transport of Cy3 labeled virus particle

To investigate possible anterograde axonal transport, Cy3 labeled baculoviruses were injected into the rat vitreous body in order to transduce the retina ganglion cells (RGC). These neurons have long axons projecting to the lateral geniculate body (LGB) and superior colliculus (SC) and forming synapses with interneurons located there. From the LGB, the axons of the interneurons fan out through the deep white matter of the brain to form the optic radiations, which will ultimately terminate in the primary visual cortex at the posterior part of the brain.

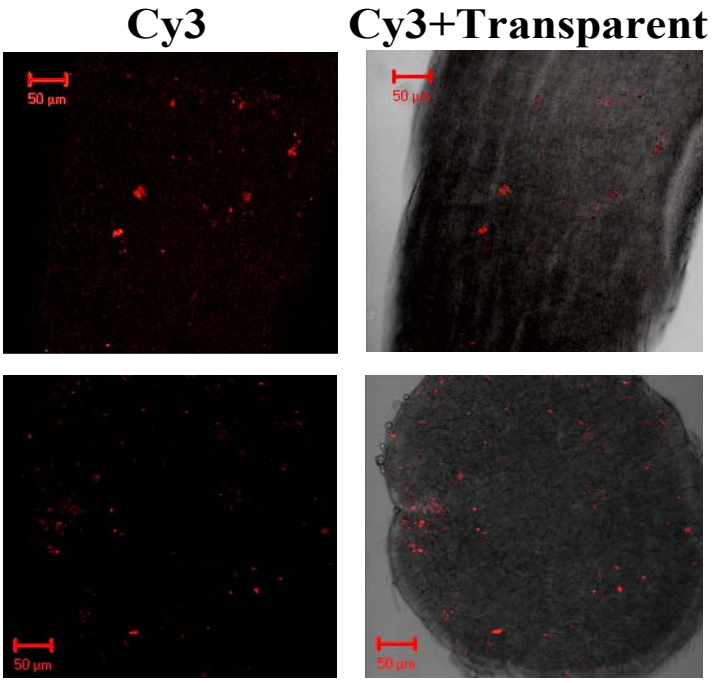
After intra-vitreous body injection of 10^9 pfu of the labeled baculovirus vectors, viral particles were observable under confocal microscope in different parts of the visual system, from the retina, the optic nerve, the LGB, the SC to the primary visual cortex (Fig. 5-5). In the retina, viral particles were detected in both NeuN-positive and negative cells. The labeled particles were detected in the optic nerve and distributed along the sagittal section of the nerve, indicating anterograde travel of the virus along the axons of the RGCs. The Cy3 signals were strictly located in NeuN-positive neurons in the LGB and the SC, indicating a possible trans-synaptic transport of the particles to post-synaptic

neurons. The hypothesis of viral transport across the synaptic cleft was strongly supported by the detection of the Cy3 signals in the tertiary neurons in primary visual cortex.

A) Retina



B) Optic Nerve



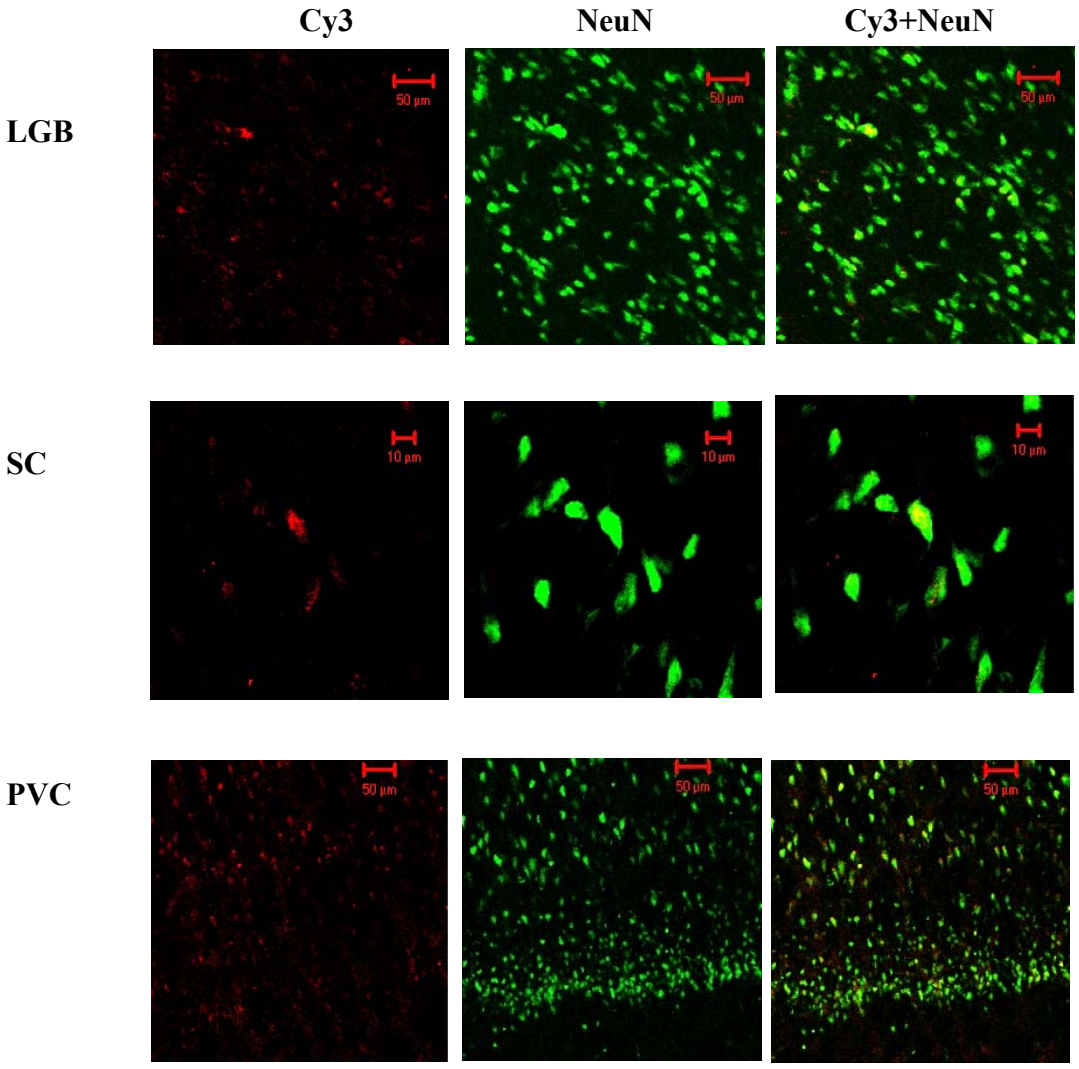


Fig. 5-5 Confocal images of uptake and transport of Cy3 labeled baculovirus by neurons after intra-vitreous body injection. Frozen coronal sections of each eye ball and brain were cut at 30µm thickness for immunostaining against neuron-specific nuclear protein (NeuN) to show neurons. Frozen cross and sagittal sections for optic nerve were used for confocal analyze directly. Scare bar present for 50µm for images of retina, optic nerve, LGB and primary visual cortex; 10µm for SC. GCL, ganglion cell layer; INL, inner nuclear layer.

5.4.2.2 Baculovirus genome detected by PCR analysis

PCR analysis of the viral DNA in the samples collected from the different parts of the visual system provided another piece of supporting evidence for the anterograde transport and trans-synaptic transported of the baculovirus vectors (Fig 5-6).

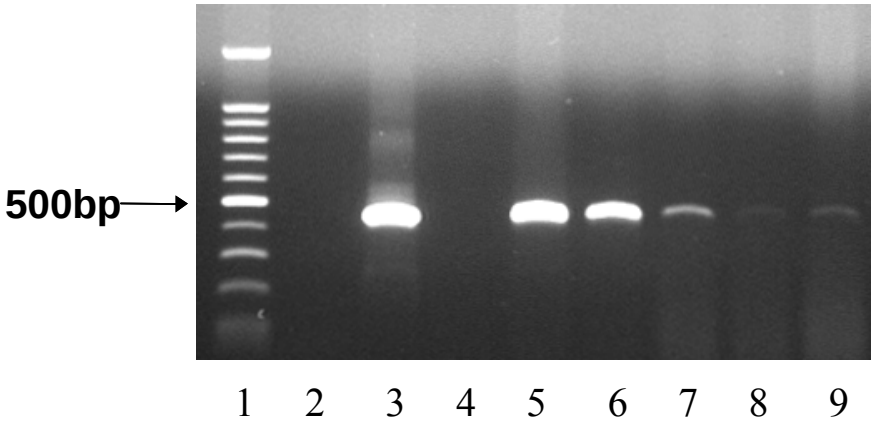


Fig. 5-6 Baculovirus genome detected by PCR in the visual system after intra-vitreous body injection. Lane 1, 100bp ladder; lane 2 & lane 4, no injection and shamed injection with 1XPBS as negative control; lane 3, purified BV-CMV E/PDGF genome as positive control; lanes 5, 6,7,8, and 9, retina, optic nerve, lateral geniculate nucleus (LGB), superior colliculus (SC), and primary visual cortex (PVC).

5.4.2.3 Reporter gene expression tested by luciferase assay

The possibility of using beculoviruses for gene transfer in projected, post-synaptic neurons was then investigated with a luciferase reporter gene vector under the control of the neuron-specific CMV E/PDGF promoter. After intra-vitreous body injection of $1x10^7$ pfu of the recombinant baculovirus, luciferase expression was detected in all parts of the visual circuit, with the highest transgene expression in the retina, followed by that in LGN and primary visual cortex. The SC gave the lowest luciferase expression, consistent with the fact that only a small amount of axons from RGCs projecting to this region in rats.

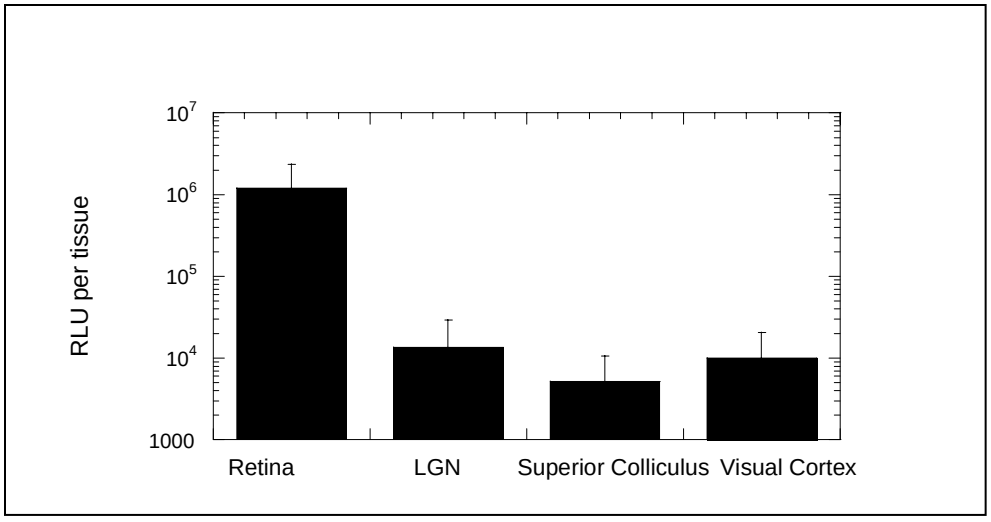


Fig. 5-7 Luciferase expression along visual pathway after intra-vitreous body injection. Four rats were used for the experiment. Values are presented as means ± SD.

5.4.2.4 Reporter gene expression localized by double staining

As revealed by immunohistological staining, luciferase expression was confined only to neurons, no matter which part of the visual system was examined (Fig 5-8). Most of the transduced neurons in the LGB were with large cell bodies (Fig. 8G, H). These are likely to be the large M cells of the LGB that receive the inputs from large ganglion cells.

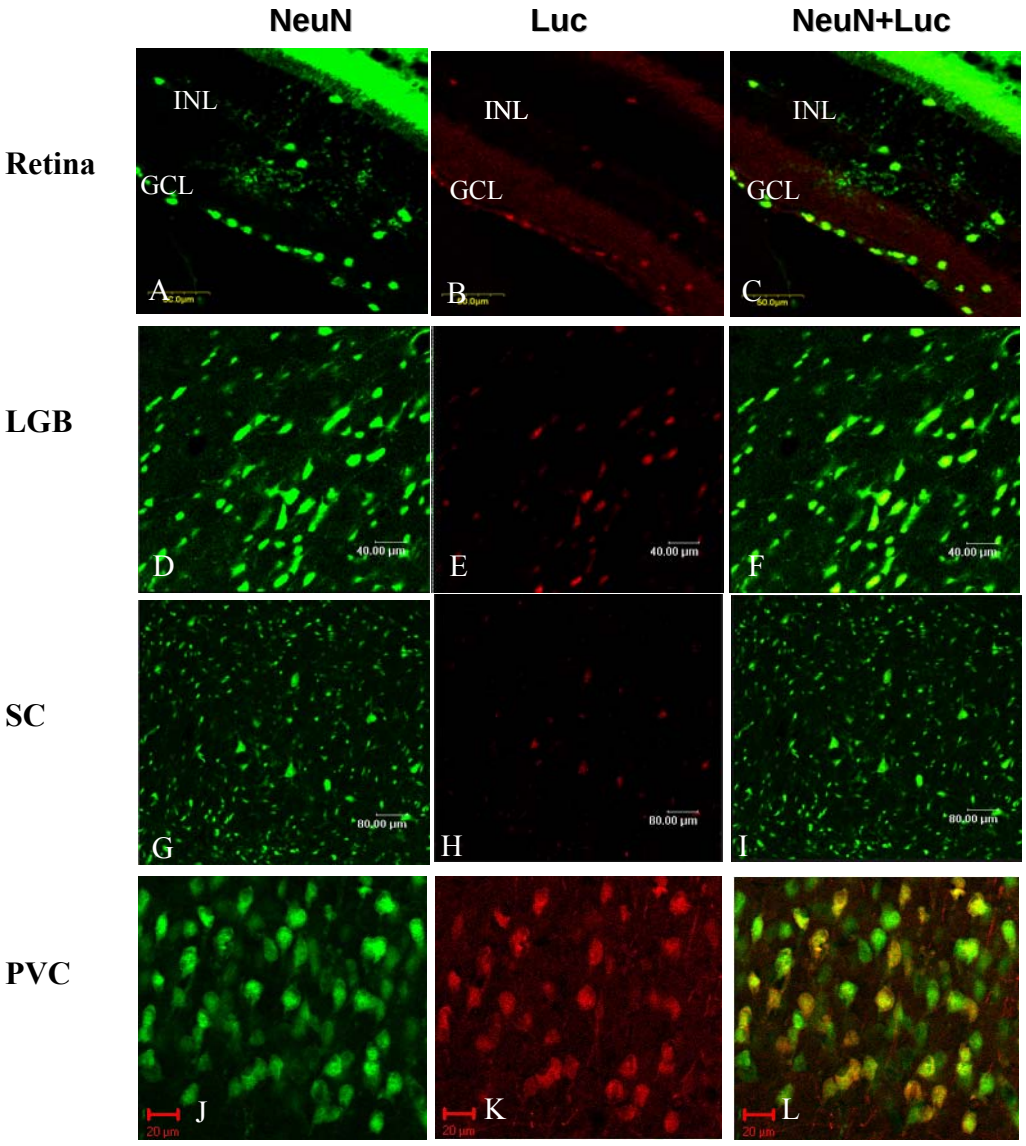


Fig. 5-8 Confocal images showing luciferase expression in neurons after intravitreous body injection. Tissue samples were collected 2 days after injection. Tissue samples were dissected the same way as previously described for double immunostaining against luciferase to show transfected cells, against neuron-specific nuclear protein (NeuN) to show neurons. Scale bar present 40um for A-F, 80um for G-I; I: Retina; II: Lateral geniculate body (LGB); III: Superior culliculus (SC); IV: Primary visual cortex (PVC).

5.5. Discussion

This study has examined the transport of baculovirus in two systems of the rat brain: the striatal system and the visual system. The question of how the virus was transported was addressed with different approaches. Firstly, florescence labeling of envelope proteins of the virus and injection of labeled viruses were used for direct visualization of viral particles. Secondly, virus genome was detected by PCR. And thirdly, luciferase reporter gene expression was detected through quantitative measurement of luciferase activity and qualitative immunostaining. The findings suggest that baculoviruses could be transported in axons and across the synaptic cleft.

Several viral delivery strategies for neuronal gene transfection made use of the axonal transport mechanism. This approach allows viral vectors to target both neurons at the injection site and the projection neurons at the distal parts of the neural circuits. By using an injection site at a more accessible brain region, the approach may target the populations of neurons deep in the CNS structure and minimize the damage to sensitive regions caused by injection procedure. The retrograde transport of AAV was observed after injection into striatum and hippocampus (Kaspar et al., 2002c), while anterograde transport of virus particles hasn't been observed so far. For another commonly used virus, adenovirus, no anterograde transport was reported, while its retrograde transport were shown by different groups after brain injection (Soudais et al., 2001b; Peltekian et al., 2002b; Mittoux et al., 2002b) and intramuscular injection(Ghadge et al., 1995a; Boulis et al., 1999b; Keir et al., 1995). Bidirectional and transneuronal transport of HSV were reported by different groups after injection into different nerve systems: anterograde and transneuronal transport in visual systems (Norgren et al., 1992a; Sun et al., 1996a);

retrograde transport in somatosensory systems (Palmer et al., 2000b; Antunes Bras et al., 1998; Yamamura et al., 2000a) and other systems (McLean et al., 1989b; Norgren et al., 1998; Blessing et al., 1994a).

According to the in vivo results of our studies, baculovirus undergoes both bidirectional and transneuronal transport similar to that of HSV. Owing to the efficient trans-synaptical transport of baculoviruses, they also have the potential to act as a potent neuronal tracer for tracing both the sensory and motory pathway. Compared to the other transneuronal tracer viruses, the anterograde axonal transport of which is usually relatively slower and less efficient than the retrograde route (Vann and Atherton 1991; Sun et al., 1996b; Norgren et al., 1992b; McLean et al., 1989a; Card et al., 1990; Card et al., 1991; Blessing et al., 1994b), baculoviruses spread quite fast within the axoplasm in both anterograde and retrograde directions, within 1 days from the striatum to the cerebral cortex and from the retina to the primary visual cortex. The viruses may, however, not be suitable for tracing those reciprocal pathways due to the bi-directional axonal transport.

The strategy of CNS gene delivery by retrograde transport after intramuscular injection was utilized to deliver several kinds of viral vectros, including adenovirus (Boulis et al., 1999a;Ghadge et al., 1995b), HSV (Palmer et al., 2000a;Yamamura et al., 2000b) and PEI/DNA complexes(Wang et al., 2001). This approach bypasses the blood-brain barrier and provides a practical therapeutic strategy that is noninvasive to CNS tissues (Wang et al., 2001). However, we failed to transduce motor neurons in hypoglossal nucleus and spinal cord after muscle injection of the baculovirus vector into the skeletal muscles of

tongue and hinder limb (unpublished observation). Another vector which also exhibit retrograde transport in CNS(Kaspar et al., 2002b) but not in PNS after muscle injection(Martinov et al., 2002), is AAV. Interestingly, the retrograde transport of AAV was only observed after injection into striatum and hippocampus (Kaspar et al., 2002a), but not after virus delivery into another nucleus, the parabranchial nucleus(Chamberlin et al., 1998). It seems that the differences of the nerve terminals between CNS and PNS neurons or among various groups of neurons in CNS may be the cause of the different transport paradigm of a viral vector.

Cytoplasmic transport of baculovirus nucleocapsid in insect cells is supposed to be a result of capsid-induced actin polymerization(Charlton and Volkman 1993;Lanier and Volkman 1998). In mammalian cells, depolymerization of actin filaments by chemicals inhibits the nuclear transport of the virus, thus inhibiting the virus-mediated transgene expression, while dypolymerization of microtubules increases the expression of reporter gene(van Loo et al., 2001). Several investigators have demonstrated that baculovirus enters mammalian cells via electrostatic interaction mediated endocytosis (Duisit et al., 1999;Tani et al., 2001), followed by cytoplasmic transport through endosomal pathway (Boyce and Bucher 1996;Hofmann et al., 1995;van Loo et al., 2001). Late endosomes are supposed to undergo fast retrograde transport in axons (Brady 1991), and the endocytic membrane system helps the endocytosed virus to maneuver through the dense actin cortex beneath the plasma membrane (Gottlieb et al., 1993). Endosomes can travel long distances with the help of motor proteins, which is particularly useful in neurons where the site of entry can be far away from the site of viral replication (Enquist et al., 1998).

Further studies should be done to elucidate that if this transport mechanism is also used by baculovirus. The interaction between microtubule motor protein, dynein or kinesin, and nucleocapsid protein of the several virus vectors capable of axonal transport has been investigated(Raux et al., 2000;Diefenbach et al., 2002). Similar studies for baculovirus would be useful for investigating the axonal transport mechanisms of this vector. Since the fluorophore, Cy3, is mainly conjugated to the envelop protein, we can speculate that the virus envolop protein such as gp64 may be involved in the interaction with some motor proteins.

With the assistance of a neuron-specific promoter, the transgene expression in the retina was confined in ganglion cells after intravitreous body injection of BV-CMVE/PDGF, although the viruses entered both neurons and non-neuronal cells in the retina as revealed by Cy3-labeled virus. This attribute may be utilized for studying and curing RGC diseases, or for reducing axotomy-induced RGC death by delivery of a therapeutic gene(Harvey et al., 2002;Weise et al., 2000).

In summary, the current study has contributed to our knowledge of baculovirus vectors and may lead to more extensive use of this virus as an effective neuronal gene delivery vector and probably also a tracer for neural circuitries. The targeted transduction of dopaminergic neurons by the vectors in the substantia nigra provides the possibility of using them for gene therapy of Parkinson's disease (Bilang-Bleuel et al., 1997a).Ttransgene expression mediated by the baculovirus vectors in neurons widely distrubuted to various regions of the cerebral cortex, which is not feasible to direct local

injection, may confer corticostriatopallidal neuroprotection to neurons, for example those affected in Huntington's disease (HD)(Mittoux et al., 2002a).

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

The first part of this study examed the bioavailability of novel polymeric carrier, PPE-EA, for CNS gene delivery. PPE-EA displayed comparable transgene expression level to that of PEI, but with much lower cytotoxicity both in cultured neural cells and mouse brain tissue. In addition, PPE-EA/DNA complexes, which act as a controlled DNA release system, is likely to be a promising candidate in CNS gene therapy. Further investigations should test therapeutic genes to verify the potential of clinical applications of PPE-EA. It is also feasible to modify PPE-EA for cell type specific gene delivery by chemically conjugating it with targeting ligands, such as NGF, BDNF, or functional peptides.

The second part of this study focused on developing new recombinant baculovirus with a neuronal specific promoter to achieve neuron-targeted transgene expression. The findings demonstrated that this new baculovirus vector offered neuronal specific and prolonged transgene expression in primary neural culture and in rat brain. One explanation could be that the cellular authentic sequences in the hybrid promoter help to stabilize the transgene expression by reducing the inactivation of the promoter, executed by the defense mechanisms in the cells. This approach establishes the baculovirus as an efficient targetable gene delivery vector in CNS. In the current study, only one neuronal specific promoter was used to drive the reporter gene expression. More neuronal specific promoters such as NSE (rat neuron-specific enolase gene promoter) and ta1 (tat tubulin α1 gene promoter) could be used as alternatives to obtain neuron-specific expression for our recombinant baculovirus vector . It will be significant to be able to narrow the gene expression in a particular type of neurons by using a promoter with more stringent

specificity, for example, TH (rat tyrosine hydroxylase) promoter for specific gene expression in dopaminergic neurons. Furthermore, therapeutic genes like NGF, BDNF, and GDNF should be used in experimental gene therapy treatment in neurodegenerative animal models, such as Parkinson's disease rat, which will provide further information on the efficacy of the baculovirus vector.

In the last part of this study the transport profile of baculovirus particles in CNS circuits was investigated. After intra-striatum injection, baculovirus particles, genomes and virus mediated reporter gene expression were detected in the pyramidal neurons of cerebral cortex and also in dopaminergic neurons of the substantia nigra. These findings strongly support the hypothesis of retrograde transport of the virus particles in striatal pathway. In the visual system, virus particles, genomes and reporter gene expression were found in RGC, LGB, SC, and even as far as primary visual cortex after intravitreous body injection. These findings were in line with the hypothesis that virus particles could not only be transported anterogradely along the axons of RGCs, but they were also transported across the synaptic cleft between neurons. Axonal transport of virus vector in CNS may facilitate targeting gene delivery to some cell populations which are inaccessible by direct administration strategies. To our knowledge, this study is the first one to investigate axonal transport of baculovirus vectors in CNS circuits. Given the broad distribution of neurons affected by neurodegenerative disorders, gene delivery to neurons through retrograde, anterograde or transneuronal transport provides for strategic therapeutic intervention at most levels of neural circuits. In this study, only two neural circuits were investigated. More neural circuits should be studied to test the feasibility

and flexibility of baculovirus in CNS gene therapy. It would be very interesting to investigate, for example, the targeted delivery of anti-apoptotic or other therapeutic genes to vulnerable projection neurons in animal model with early stage neurodegenerative diseases such as Parkinson's disease.

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