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IN VIVO PROTEIN TRANSDUCTION TO THE CNS

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Abstract—Proteins and peptides are useful research and therapeutic tools, however applications are limited because delivery to the desired location is not easily achievable. There are two hurdles in protein/peptide delivery to the brain: the blood–brain barrier and intracellular penetration. Penetration to both brain and the intracellular space can be achieved by adjusting hydrophilicity, and small molecule pharmacological agents have been successfully developed using this approach. But with proteins and peptides, it is difficult to modify the hydrophilicity without influencing biological functions. Trans-acting factor protein from the human immunodeficiency virus contains a highly conserved cationic peptide sequence necessary for transduction across the cell membrane. While trans-acting factor peptide has been used for *in vitro* protein transduction, its *in vivo* application is very limited because it is rapidly degraded by proteolysis. Polyethylenimine is a chemically synthesized small molecule cationization agent; the charge density is greater than a peptide-based cationic cluster such as trans-acting factor, and it is resistant to proteolysis *in vivo*. We first tested intracellular protein transduction following direct brain injection in mice using polyethylenimine-conjugated green fluorescence protein and β-galactosidase (molecular weights 29 and 540 kDa, respectively). Polyethylenimine-conjugates penetrated to the intracellular space immediately surrounding the injection site within one hour. We further tested polyethylenimine-mediated protein transduction following intranasal administration, which bypasses the blood–brain barrier. Polyethylenimine-conjugates in pH 7.5 solution did not reach the brain, probably because the polyethylenimine-conjugates penetrated into the intracellular space where first exposed to the tissue, i.e. at the nasal mucosae. We temporarily reduced the electrostatic interaction between cationized polyethylenimine-conjugates and cellular surfaces by adjusting the pH to 4.5; solution rapidly reached the brain and penetrated to the intracellular space. This study suggests that polyethylenimine is a useful protein transduction agent in the brain *in vivo*, and adjusting cationic charge interaction can determine the extent of brain penetration.

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Abbreviations: β-Gal, β-galactosidase; GFP, green fluorescence protein; PBS, phosphate-buffered saline; PEI, polyethylenimine; Tat, trans-acting factor.

Key words: protein transduction, polyethylenimine, intranasal delivery, trans-acting factor (Tat), blood–brain barrier, drug delivery.

In vivo protein transduction, the delivery of proteins into living cells, has many potential applications, ranging from basic experimentation to the development of novel therapeutics. However, proteins and peptides do not penetrate into cells to any great degree unless an active transporter is in place. Trans-acting factor (Tat) was identified as a highly conserved sequence among different human immunodeficiency viruses (HIV) (Endo et al., 1989). Tat contains a highly cationic arginine and lysine cluster, and an N-terminal alpha helical structure (Loret et al., 1991). Conjugation to Tat peptide can be used to deliver large proteins, such as RNase, domain III of pseudomonas exotoxin A, horseradish peroxidase and β-galactosidase (β-Gal) (MW=13.6, 30, 40 and 540 kDa, respectively) into the intracellular space (Fawell et al., 1994). In addition, Tat-mediated protein transduction into living cells leads to biological effects (Nagahara et al., 1998; Gius et al., 1999). The cationic cluster, which consists of six arginines and two lysines within a 9-amino acid linear sequence, was identified as the protein transducting domain (Vives et al., 1997; Futaki et al., 2001; Kramer and Wunderli-Allenspach, 2003). The detailed mechanism of Tat-mediated protein transduction is not yet fully understood, but the cationic cluster is essential (Brooks et al., 2005). Polyethylenimine (PEI) is a polymer with a branched backbone of two carbons followed by one potentially protonated nitrogen in every monomer mass unit of 43. This charge density compares very favorably with the equivalent mass sizes of 157 for arginine and 129 for lysine in aqueous solution. More importantly, PEI is a chemically synthesized small molecule and resistant to protease degradation (Futami et al., 2005), while Tat is a peptide and is thus vulnerable to protease degradation (Lundberg et al., 2003). PEI has been used for *in vitro* oligonucleotide transduction (Lemkine and Demeneix, 2001; Ohashi et al., 2001) and *in vitro* protein transduction (Futami et al., 2005; Kitazoe et al., 2005). Toxicity studies showed that PEI has a wide safety margin; mouse LD_{50} is 1.14 g PEI/kg, which is approximately 10,000-fold higher than the amount used for *in vivo* transduction during this study.

While the precise dynamics of intranasal delivery of substances to the CNS are still unclear, a wide variety of molecules are capable of moving from regions of the olfactory nerve dendrites in the olfactory epithelium, through

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the cribriform plate into the brain (Balin et al., 1986; Thorne et al., 1995). The primary advantage of intranasal delivery is that this non-invasive method bypasses the blood–brain barrier, which limits the entry of large molecules to the CNS. Moreover, this approach has potential to deliver small molecules that would be rapidly removed from the bloodstream by hepatic first pass elimination. The intranasal delivery of proteins, peptides and small molecules to the CNS is an area of emerging interest, with recent publications documenting entry to the brain of substances as diverse as insulin-like growth factor-1 (Thorne et al., 2004), vasoactive intestinal peptide (Dufes et al., 2003) and 17 beta-estradiol prodrugs (Al-Ghananeem et al., 2002). These molecules are delivered to the extracellular space in the brain, and act through receptors located on the cell membrane. While intranasal administration bypasses the blood–brain barrier, transduction to the intracellular space is required when the point of action is in the intracellular space.

In this study, we aimed to develop a non-invasive method to deliver large proteins to the intracellular space. We used green fluorescence protein (GFP) and β-Gal without or with PEI conjugation as proof-of-principle molecules, and examined cellular penetration through direct and intranasal administration.

EXPERIMENTAL PROCEDURES

Recombinant protein preparation

Hexahistidine-tagged enhanced GFP was expressed using UV5C48Stag:GFPuv5tag mutations plus C48S vector (Suzuki et al., 2004) and purified as previously described (Futami et al., 2005). PEI with an average molecular weight of 600 (Nippon Syokubai, Osaka, Japan) was coupled with GFP or avidin as previously described (Yamada et al., 1981; Kitazoe et al., 2005, respectively). Biotinylated β-Gal (Sigma, St. Louis, MO, USA) was conjugated with avidin-coupled PEI in phosphate-buffered saline (PBS) for 1 h at 1:8 molar ratio prior to use.

Direct administration to the brain and tissue preparation

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Georgetown University and followed guidelines set forth in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals." ICR mice (Harlan, Madison, WI, USA) at 10–13 weeks of age were immobilized in a mouse stereotaxic frame under anesthesia (chloral hydrate, 50 mg/kg, i.p.). Compounds were injected into the cerebral cortex (−1.5 mm from Bregma, 0.5 mm lateral, 1.5 mm depth from the skull) or striatum (−0.8 mm from the Bregma, 2.5 mm lateral, 3.5 mm depth from the skull) using a Hamilton syringe. We used two proof-of-principle compounds without or with PEI conjugation; GFP ($n=7$ in each group, 2 μl of 7.8 μg GFP/μl), and β-Gal ($n=5$ in each group, 2 μl of 4.02 μg β-Gal/μl). For control, same volume of vehicle, 30 mM HEPES–NaOH buffer, pH 7.5, containing 0.1 M NaCl, was given ($n=4$). The animals were killed after 1 h, the brain was quickly isolated, and each hemibrain was subjected to histochemical and biochemical assessments. For histological assessment, brains from mice that received GFP or PEI-GFP were immersion fixed in 2 or 4% paraformaldehyde for 24 h, then dehydrated in 15% sucrose in 100 mM phosphate buffer (PB), pH 7.4. Brains that received β-Gal were cut into 1–2 mm thickness slice and then immersion fixed in 4% paraformaldehyde for a shorter period, 2 h, to preserve its enzymatic activity, then dehydrated for 15 h. For biochemical analysis, hemibrains were snap frozen in dry ice and stored at −80 °C. Thirty micrometer-thickness sagittal sections were prepared using a cryostat.

Intranasal administration and tissue preparation

ICR mice at 10–13 weeks of age were anesthetized with chloral hydrate (50 mg/kg, i.p.) 15 min prior to intranasal administration. Total of 20 μl of PEI-GFP, GFP (both 7.8 mg/ml, $n=9$ each) or vehicle ($n=3$) was administered in 5 μl per nostril every 2 min. GFP and PEI-GFP were prepared in two different buffers: 30 mM HEPES–NaOH buffer, pH 7.5, containing 138 mM NaCl; and 100 mM unbuffered potassium phosphate monobasic, pH 4.5, containing 138 mM NaCl. After one hour, mice were perfused with 20 ml of 10 mM PBS, pH 7.4, under anesthesia. Brains were isolated and prepared for histochemical and biochemical assessments as described above.

In vivo analysis

GFP fluorescence was observed under a fluorescence microscope (excitation: 465–495 nm, emission: 515–555 nm) and images were captured by digital camera (SPOT RTke, SPOT Diagnostics, Sterling Heights, MI, USA). GFP-positive neurons in 1 mm square surrounding the injection site were counted, and the percentage of GFP-positive neurons among all morphologically identified neurons (Duchen, 1992) using Cresyl Violet staining was determined. Sections were counterstained with a nucleic acid-specific dye, TO-PRO-3 (excitation: 642 nm, emission: 661 nm, Invitrogen, Carlsbad, CA, USA) at 1 μM. For three-dimensional analysis, sections were analyzed by a confocal microscope (LSM510, Zeiss, Jena, Germany). β-Gal activity was visualized by incubation at 37 °C for 8 h in a solution consisting of 1 mg/ml X-Gal (Fisher Biotech, Fairlawn, NJ, USA), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS.

For quantitative measurement of fluorescence, the hemibrain was homogenized in 10-fold volume of Tris–HCl buffer, pH 7.6 containing 250 mM sucrose, 1 mM EDTA and EGTA, and protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 45,000×*g* for 30 min, and the supernatant was used for fluorescence detection (excitation: 485 nm, emission: 535 nm, Wallac Victor III, Perkin Elmer, Wellesley, MA, USA). To quantify CNS entry, GFP or PEI-GFP were serially diluted in mice brain homogenate prepared from untreated mice and the standard curve was drawn.

In vitro protein transduction

SH-SY5Y neuroblastoma cells (Biedler et al., 1978) were cultured using a chamber slide (BD Falcon, Franklin Lakes, NJ, USA) in OptiMEM (Invitrogen) with 10% fetal bovine serum. GFP without or with PEI conjugation (225 μM) were in 30 mM HEPES–NaOH buffer, pH 7.5, containing 0.1 M NaCl, and added to semi-confluent cells at 1:100 dilution to yield the final concentration of 2.25 μM for 24 h. Thus, cell treatment was carried out at neutral pH. After incubation, fluorescence were observed prior to the fixation and after fixation with 2% paraformaldehyde for 10 min.

RESULTS

PEI-mediated GFP transduction to the intracellular space *in vivo*

GFP (MW=29 kDa) without or with PEI conjugation were injected into the cerebral cortex. One hour after the injec-

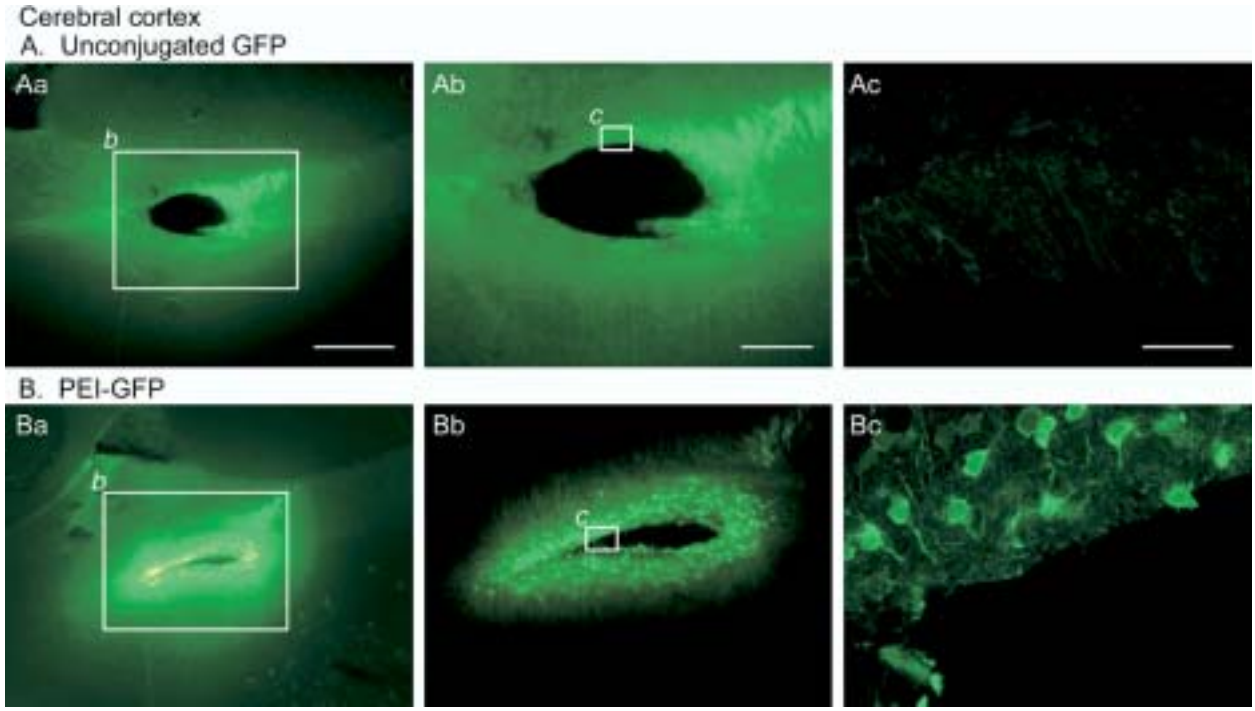

Fig. 1. Unconjugated GFP, MW=29 kDa, (A) and PEI-conjugated GFP, MW=29 kDa (GFP) plus 0.6 kDa (PEI), (B) were injected to the cerebral cortex and the GFP fluorescence was observed. Area indicated by box in panels Aa/Ba and Ab/Bb is shown in Ab/Bb and Ac/Bc at higher magnification, respectively. While PEI-GFP penetrates to the intracellular space, unconjugated GFP showed very limited penetration. Scale bar=500, 200 and 50 μm in panels Aa for Aa and Ba, Ab for Ab and Bb, and Ac for Ac and Bc, respectively.

tion, the brain was quickly isolated and fixed, and GFP distribution and subcellular localization were examined. Unconjugated GFP diffused widely around the area injected, but did not penetrate into the intracellular space (Fig. 1A). PEI-GFP distributed similarly in the extracellular space (Fig.1Ba), and, notably, PEI-GFP penetrated into the intracellular space immediately surrounding the injection site (Fig. 1Bb and 1Bc). We also injected the compounds into a deeper brain region, the striatum, and found the same results: PEI-GFP showed much greater cellular penetration (Fig. 2B) compared with unconjugated-GFP (Fig. 2A). PEI-mediated GFP transduction to the intracellular space was limited to the area immediately surrounding the injection site. Morphological characteristics of GFP-positive cells indicate that they are neurons (Duchen, 1992). Approximately 70% of the neurons in 1 mm square surrounding the injection site were observed with GFP. Some PEI-GFP penetrated into the intranuclear space, which was visualized by TO-PRO-3, a nucleic acid-specific dye (Fig. 3). We compared subcellular localization using 2 and 4% paraformaldehyde-fixed tissue, and no difference in subcellular distribution was found between the two concentrations of fixative (data not shown).

We further tested a much larger protein, β-Gal (MW=540 kDa), as a second proof-of-principle compound. β-Gal and PEI-β-Gal were injected in the same manner as GFP, and β-Gal was visualized by enzymatic reaction. PEI-β-Gal distributed to a wider area (Fig. 4B) compared with unconjugated β-Gal, which diffused in very limited area (Fig. 4A). Number of β-Gal positive cells is significantly high in mice receiving PEI-GFP (insets in Fig. 4A and B).

Intranasal administration of PEI-GFP penetrates into the intraneuronal space in the brain

GFP without or with PEI conjugation was prepared at neutral pH, 7.4, and intranasally administered. GFP fluorescent measurement in the brain homogenate determined that approximately 1–2% of the administered unconjugated GFP entered the CNS (the detection limit of the method is 0.3% CNS entry), which is in agreement with previously published results (Thorne et al., 2004). Fluorescence was histochemically invisible following intranasal administration of unconjugated GFP (data not shown). The fluorescence level in brain homogenate derived from PEI-GFP-treated mice was below the detection limit (i.e. less than 0.3% CNS entry), and immunoblot using anti-GFP antibody confirmed these results (data not shown), indicating that PEI-GFP (pH 7.5) did not enter the brain. Histological study did not detect fluorescence following PEI-GFP (pH 7.5) administration (Fig. 5A). Since PEI-mediated protein transduction occurred immediately surrounding the injection site in the case of direct administration (Figs. 1B and 2B), we suspected that PEI-mediated protein transduction occurred in nasal tissue prior to entry into the brain. We temporarily reduced the electrostatic interaction between PEI-GFP and the cellular surface by lowering solvent pH to 4.5. Intranasally administered PEI-GFP (pH 4.5) reached the brain (Fig. 5B) and penetrated into the intra-

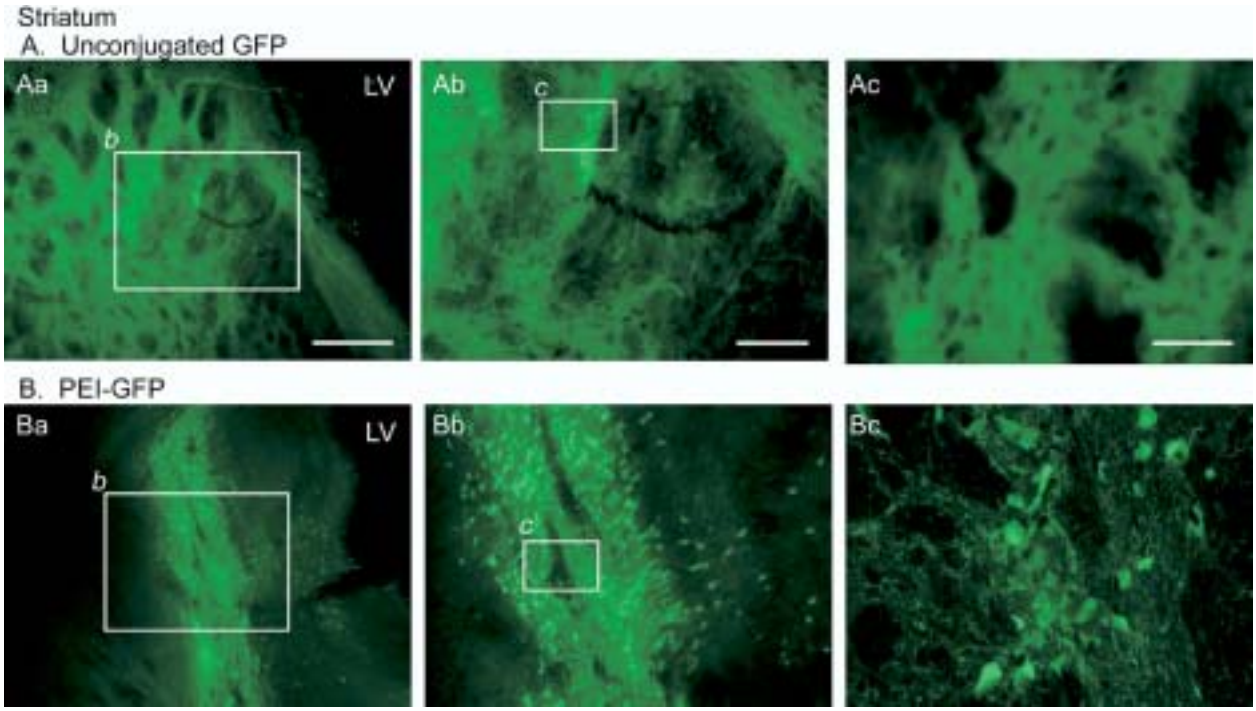

Fig. 2. Unconjugated GFP, MW=29 kDa, (A) and PEI-conjugated GFP, MW=29 kDa (GFP) plus 0.6 kDa (PEI), (B) were injected to the striatum and GFP fluorescence was observed. Area indicated by box in panels Aa/Ba and Ab/Bb is shown in Ab/Bb and Ac/Bc at higher magnification, respectively. While PEI-GFP penetrates to the intracellular space, unconjugated GFP showed virtually no cellular penetration. Scale bar=500, 200 and 50 μm in panels Aa for Aa and Ba, Ab for Ab and Bb, and Ac for Ac and Bc, respectively. LV: lateral ventricle (in panels Aa and Ba).

cellular space (Fig. 5Bb and 5Bc). We also tested intranasal treatment of unconjugated GFP at pH 4.5, but it was not histologically detectable in the brain (data not shown).

PEI-mediated protein transduction in neuron-like cells *in vitro*

While it is possible that fixation may alter cellular localization of the protein, histological examination of the unfixed brain tissue is difficult due to high background fluorescence. PEI-mediated protein transduction was previously reported in fibroblasts in culture (Futami et al., 2005); however, feasibility of protein transduction in neuron-type cells has not yet been examined. In this study, we examined PEI-mediated protein transduction in neuron-like cells *in vitro* using neuroblastoma cells, SH-SY5Y. Unconjugated GFP (Fig. 6A) does not penetrate to the intracellular space, while PEI-GFP significantly penetrates to the intracellular space (Fig. 6B). Both PEI-GFP-treated living (Fig. 6Ba) and fixed (Fig. 6Bb) cells showed significant fluorescence in the intracellular space; therefore, fixation does not notably alter localization.

DISCUSSION

Tat, the best known and most studied protein transduction domain, consists of six arginines and two lysines within a nine amino acid linear sequence (Vives et al., 1997). It has been proposed that ionic interactions between this cationic

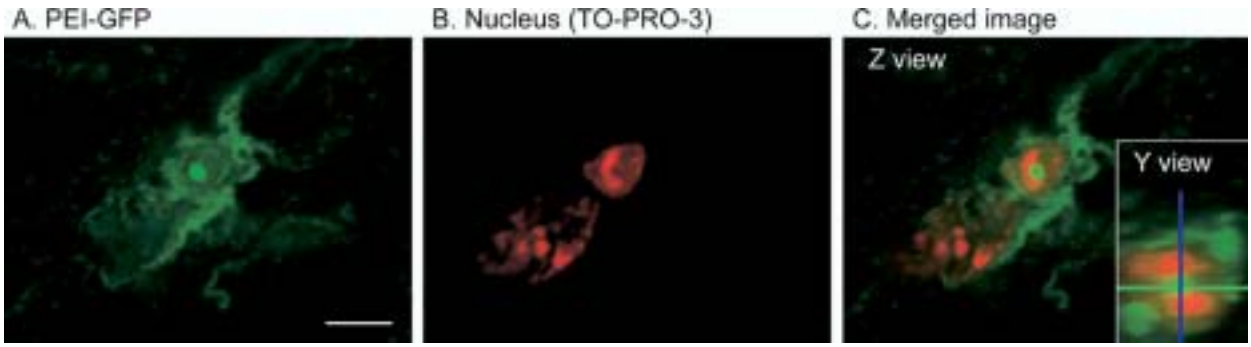

Fig. 3. PEI-GFP was injected into the cerebral cortex and subcellular localization of PEI-GFP penetration was examined by confocal microscopy (green, A). Sections were counterstained with nucleus-specific dye, TO-PRO-3 (red, B), and overlaid with GFP signals (C). The inset (C) is an orthogonal projection. Some PEI-GFP penetrates to the nucleus. Scale bar=15 μm.

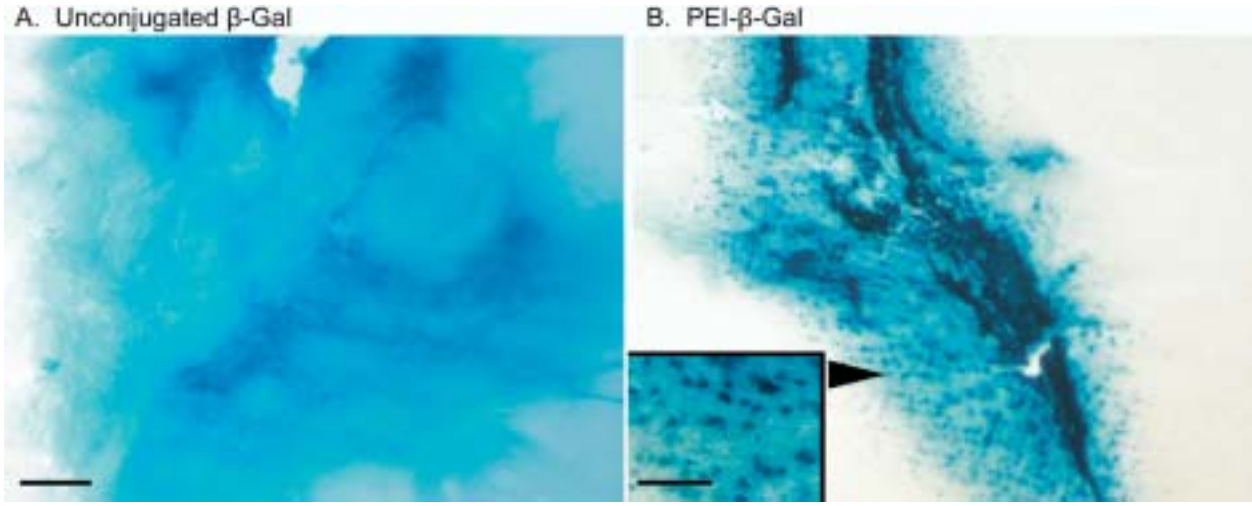

Fig. 4. β-Gal (MW=540 kDa) without (A) or with PEI conjugation (B) was injected into the striatum and distribution was visualized by enzymatic staining. While unconjugated β-Gal was evident in the extracellular space, virtually no cellular penetration was observed (A). PEI-β-Gal penetrated efficiently into the intracellular space (B). Scale bar=100 and 25 μm for main panels and insets, respectively.

cluster and the anionic phospholipid heads of the membrane lead to initial cellular binding, and that internalization occurs via an active transport mechanism involving endocytosis (Richard et al., 2003). While Tat is well characterized and has proven useful for mediating protein transduction *in vitro* (Vives et al., 1997), Tat is a peptide and is subject to proteolysis in biological environments (Lundberg et al., 2003). PEI is a chemically synthesized small molecule; it is safe and resistant to proteolysis. Furthermore, the cationic charge density of PEI (one charge/43 molecular mass) is three- and 3.6-fold higher than polyarginine and polylysine (one charge/157 and 129 molecular mass), respectively; thus PEI may be a more efficient and flexible agent for protein transduction. This study has demonstrated that PEI conjugation is highly effective in rapidly (within 1 h) inducing protein transduction in brain, being capable of transducing two functional proteins of widely differing molecular weights (MWs of GFP and β-Gal are 29 and 540 kDa, respectively) into the intracellular space. PEI mediated protein transduction in non-neuronal cells, such as murine and human fibroblasts (Futami et al., 2005). Our study showed that PEI-mediated protein transduction is also useful in neuronal cells both *in vivo* and *in vitro*. We also confirmed that our finding, protein transduction, is not due to fixation-induced artifact.

The primary advantage of intranasal delivery is that this non-invasive method bypasses the blood–brain barrier, which normally limits the entry of large molecules to the CNS. Approximately 1–3% of intranasally administered insulin-like growth factor (MW=7.65 kDa) enters the CNS, and autoradioautography indicates wide distribution throughout the entire brain (Thorne et al., 2004). Intranasal administration is an optimal brain delivery method for brain-impermeable molecules acting through receptors on the cell surface. However, bypassing the blood–brain barrier by intranasal administration may not be sufficient for molecules that act in the intracellular space. We combine the advantages of intranasal administration and PEI-mediated protein transduction in order to deliver proteins into the intracellular space of the CNS. We intranasally administered unconjugated GFP and found 1–2% CNS entry by biochemical assessment, but the distribution of GFP fluorescence was not histologically visible. We tested intranasal administration at pH 4.5 and 7.5. Brain entry of unconjugated GFP was not histologically detectable at either pH 4.5 and 7.5. While PEI-GFP (pH 7.5) did not enter the CNS, PEI-GFP (pH 4.5) reached the brain and penetrated into the intracellular space.

Cellular membranes might be altered in an acidic environment (Fittipaldi et al., 2003). In experiments with acidic solutions, the pH is 4.5 at initial tissue exposure, i.e. at the nasal mucosa. As the intranasally administered agent is transferred to the brain, the pH is presumably adjusted to neuronal pH through physiological buffering. Therefore, acidic pH-induced cellular membrane alteration might contribute to intracellular penetration in the nasal mucosa to some degree, but we suspect that it does not play a significant role in the brain. PEI-GFP directly injected into the cerebral cortex and striatum penetrated to the intracellular space to a great degree, suggesting that transduction occurred immediately surrounding the administration site. We therefore hypothesized that brain penetration of intranasally administered PEI-GFP at neutral pH was being limited by local absorption in nasal tissue. To minimize this local adsorption, we temporary reduced the negative charge of cellular surface by lowering the pH to 4.5, which could lead to protonation of carboxyl groups in acidic proteins or carbohydrates. Intranasally administered PEI-GFP (pH 4.5) entered the brain and penetrated into the intracellular space. We conclude that adjusting the negative charge on the molecule can optimize remote tissue delivery.

Biologically active peptides/proteins have potential applications if they can be delivered to the desired location. Unless proteins/peptides are substrates of active transporters at the blood–brain barrier, they are poorly permeable into the

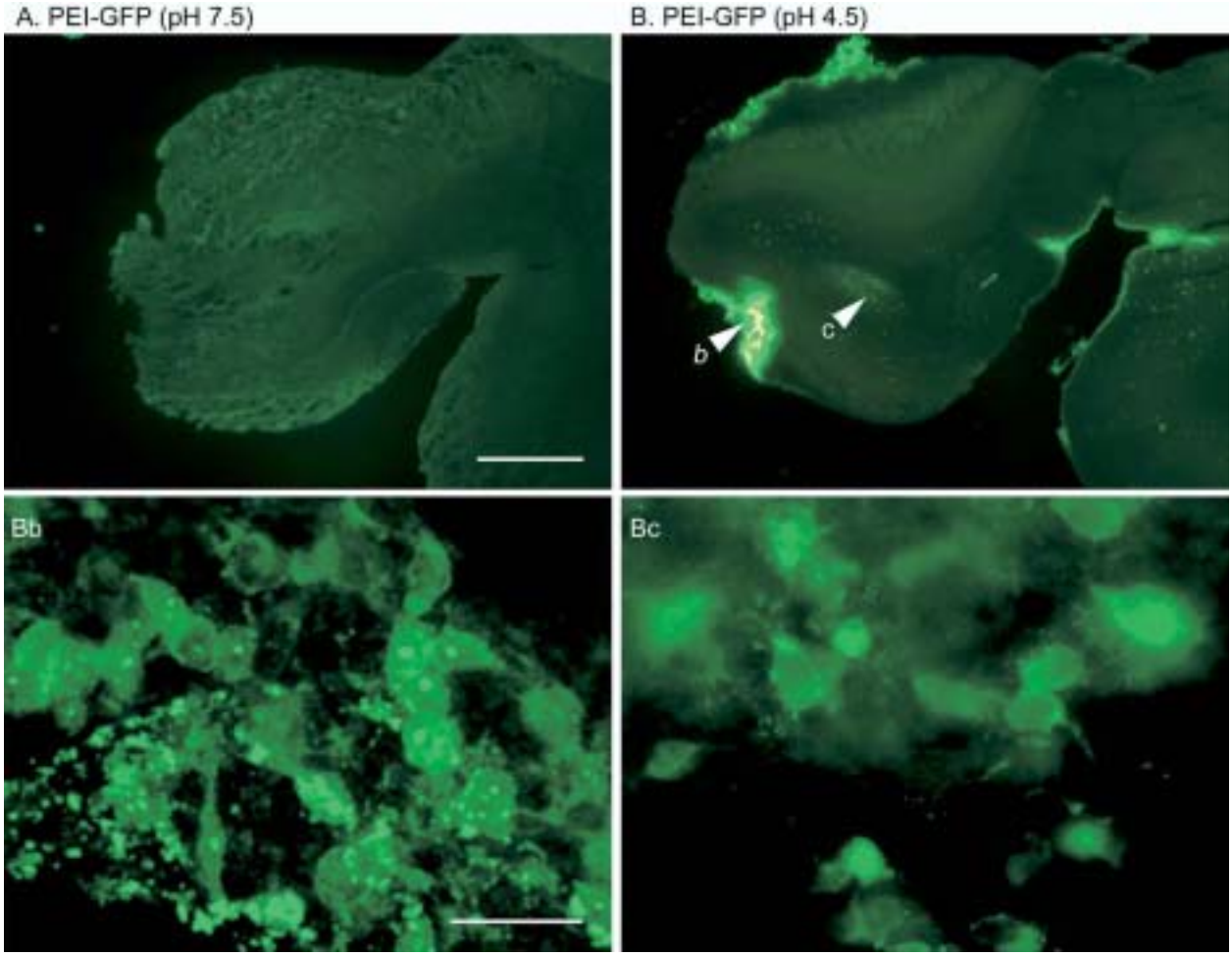

Fig. 5. PEI-GFP prepared in solution at pH 7.5 (A) or pH 4.5 (B) was intranasally administered, and distribution was observed. While PEI-GFP (pH 7.5) did not enter the brain, PEI-GFP (pH 4.5) entered the brain and penetrated to the intracellular space. The olfactory bulbs were shown at lower magnification, and areas indicated by arrowheads (B) are shown in Bb and Bc. Scale bar=500 and 25 μm for A and B, and Bb and Bc, respectively.

brain. Intranasal delivery is optimal if molecules act through receptors on the cell surface. However, intranasal administration alone is not sufficient for compounds acting in the intracellular space. This study found that PEI conjugation is a highly effective method of inducing protein transduction in neural tissue *in vivo*. Additionally, PEI can be used

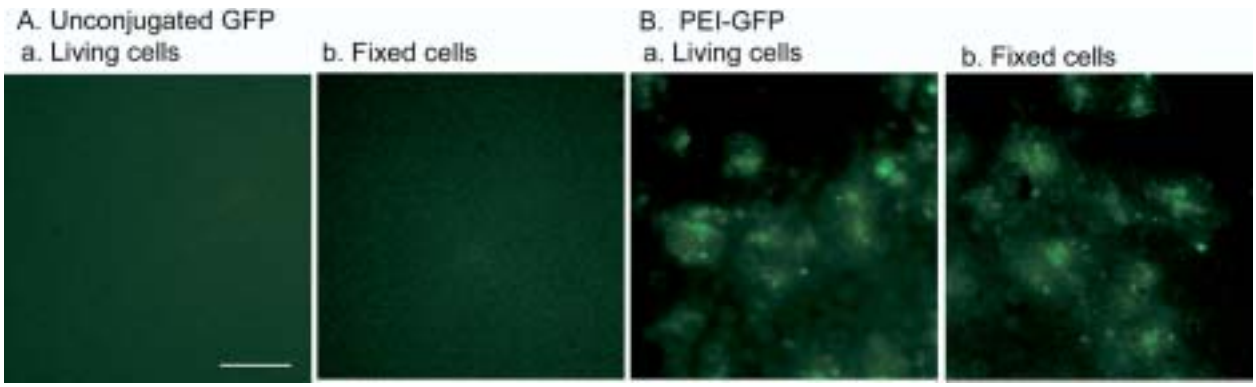

Fig. 6. PEI-mediated *in vitro* protein transduction. Unconjugated GFP (A) and PEI-conjugated GFP (B) were incubated with SH-SY5Y neuroblastoma cells for 24 h. PEI-conjugated GFP showed intracellular fluorescence (B). Fluorescence was observed prior to fixation (Aa and Ba) and after fixation with 2% paraformaldehyde (Ab and Bb). Please note that the culture slide was removed from the microscope and fixed after imaged panels; the location is not matched between panels Aa/Ab and Ba/Bb. Scale bar=25 μm.

in the intranasal CNS delivery of functionally active proteins with enhanced transductive properties. The development of such non-invasive delivery systems is of great importance in the future study and treatment of a variety of CNS disorders.

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