

CTb targeted non-viral cDNA delivery enhances transgene expression in neurons

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Abstract

Background Efficient neuronal gene therapy is a goal for the long-term repair and regeneration of the injured central nervous system (CNS). We investigated whether targeting cDNA to neurons with cholera toxin b chain conjugated non-viral polyplexes led to increased efficiency of non-viral gene transfer in the CNS. Here, we illustrate the potential for this strategy by demonstrating enhanced transfection of a differentiated neuronal cell type, PC12.

Methods *In vitro* transfection efficiency of a cholera toxin b chain–poly(D-lysine) molecular conjugate (CTb-K₁₀₀) was compared by fluorescence-activated cell sorting (FACS) analysis of green fluorescent protein (GFP) expression and luminometric measurement of β -galactosidase (β -gal) expression, to untargeted poly(D-lysine) (K₁₀₀) in undifferentiated and NGF-differentiated PC12 cells.

Results Transfection of undifferentiated PC12 cells with CTb-K₁₀₀ polyplexes resulted in a 36-fold increase in levels of pCMV-DNA_{LacZ} expression and a 20-fold increase in the frequency of transduction with pCMV-DNA_{GFP}, compared with untargeted K₁₀₀ polyplexes. Treatment of PC12 cells with 50 ng/ml/day of NGF for 14 days led to differentiation to a neuronal phenotype. Transfection of NGF-differentiated cells with CTb-K₁₀₀ polyplexes resulted in a 133-fold increase in levels of pCMV-DNA_{LacZ} expression and a 11-fold increase in the percentage of cells transduced with pCMV-DNA_{GFP}, compared with untargeted K₁₀₀ polyplexes. Transfection was dependent on CTb, with CTb-K₁₀₀-mediated transfections competitively inhibited with free CTb in both PC12 phenotypes.

Conclusions Non-viral systems for gene transfer in damaged CNS show superior toxicological profiles to most viruses but are limited by inefficient and non-selective gene expression in target tissue. Cholera toxin is known to interact preferentially with neuronal cells of the central and peripheral nervous systems, mediating binding through the b subunit, CTb, and the pentasaccharide moiety of the gangliosaccharide, GM1, which is present at high levels on the neuronal cell surface. Here, we show that a molecular conjugate of the CTb subunit, covalently linked to poly(D-lysine), is able to successfully target and significantly enhance transfection of a neuronal cell type, NGF-differentiated rat PC12 pheochromocytoma cells. This observation encourages the further development of non-viral strategies for the delivery of therapeutic genes to neurons. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords non-viral; gene transfer; central nervous system; cholera toxin; PC12 cells

Introduction

The inability of the central nervous system (CNS) to regenerate following CNS trauma results in axonal degeneration and neuronal death. To ameliorate the sequelae of *in vivo* post-injury events a number of different approaches have been adopted. Previously, we have shown that implantation of a peripheral nerve (PN) into the vitreal body of the eye rescues retinal ganglion cells from death and stimulates axonal regeneration after optic nerve transection [1–3]. PN-derived neurotrophic factors (NTF) are thought to be responsible for regeneration in this paradigm; however, although the regeneration observed was robust, the secretion of NTF from the implant and the growth response elicited was transient. Although repeated administration of recombinant NTF into the injured CNS is an attractive therapeutic strategy, cannulation and multiple injection of animals lead to infection and precludes this approach. Gene therapy offers the possibility of long-term NTF protein expression, that is required for neuronal survival and axonal regeneration following injury, through the delivery of genes encoding NTF to neuronal cell bodies by cDNA uptake from lesion sites in projection tracts and retrograde transport along severed axons. Retrograde transport of cDNAs encoding NTF to the neuronal somata, where expression occurs, avoids the entrapment of growing axons by high concentrations of recombinant NTF delivered to the site of injury. CNS transfection has been an active area of research, with several types of viruses used due to their efficient transduction rate [4]. Although there has been some success with these viral approaches, the silencing of transgene expression, the focal immune response and the safety concerns of integrating viruses may limit the transfer of these vectors into the clinic for long-term therapeutic application of gene therapy in patients.

The need for a well-defined, safe gene delivery system for the CNS has prompted us to evaluate non-viral gene transfer as an alternative. Non-viral gene transfer has been previously explored in the CNS, with the main focus of research centred around the application of polyethylenimine (PEI); however, the transduction rates observed are low and variable [5–8]. There has been some interest in the application of gene targeting ligands in the CNS, for example, using the H_c fragment of tetanus toxin [9] and monoclonal antibodies tethered to liposomes [10], but again the results are variable and the efficiency of transfection efficiency is relatively low compared with viruses.

CTb has been used extensively in neuroscience research as a neuronal tracer [11–17]. Using the non-toxic pentameric CTb chain of the *Vibrio cholerae* bacterium [18], which specifically binds to the pentasaccharide moiety of the ganglioside GM1 [19,20], we have manufactured a novel CTb molecular conjugate coupled to a polycationic polymer poly(D-lysine), of 100 lysine residues long (K₁₀₀), to generate a polycationic non-viral gene delivery system (CTb-K₁₀₀). Here, we show GM1-specific and enhanced CTb-mediated gene transfer and

expression of two reporter gene systems in a differentiated neuronal cell line.

Materials and methods

Conjugation of CTb to poly(D-lysine)

Several different forms of CTb-K, at a CTb:K molar ratio of 1 : 1, were manufactured as previously described: CTb-K₁₃, CTb-K₃₉, CTb-K₈₄, CTb-K₁₀₀, CTb-K₁₅₂, and CTb-K₂₆₅ [39]. Briefly, CTb (Calbiochem, Notts, UK) and K₁₀₀ (Sigma, Poole, UK) were both modified with the heterobifunctional reagent *N*-succinimidyl-3-(pyridyldithio)propionate (SPDP, Sigma), desalted and the CTb-PDP reduced with dithiothreitol (Sigma). Reduced CTb was added to PDP-derivatised K₁₀₀ overnight at 4 °C. The conjugation reaction was purified on a Resource-S column (Pharmacia, Uppsala, Sweden) using a 0.15–3.0 M NaCl gradient [21]. Transfection and cell toxicity data revealed that, from the different length polymers, CTb-K₁₀₀ gave the optimal combination of transgene expression and cell survival (data not shown), and was therefore selected as a candidate for further evaluation.

Expression vectors

Mammalian expression vectors pCMV-DNA_{LacZ} and pCMV-DNA_{GFP} under the control of the CMV promoter were purchased from Clontech (Berkshire, UK). Plasmid DNA was isolated using a Qiagen endotoxin-free plasmid kit according to the manufacturer's protocol (Qiagen, Sussex, UK). Integrity and purity of isolated plasmid was analysed by agarose gel electrophoresis.

Ethidium bromide exclusion assay

Condensation of plasmid DNA by polycations was determined using an ethidium bromide exclusion assay [22] measured with a Perkin-Elmer LS-50B fluorimeter (Perkin-Elmer, Bucks, UK) at 510 nm excitation, 590 nm emission with a 515 nm cut-off filter in place. Plasmid DNA was added at a concentration of 20 mg/ml with ethidium bromide at a concentration of 400 ng/ml and an initial reading taken. CTb-K₁₀₀ or K₁₀₀ was then added in 0.013 N : P aliquots mixed briefly, then a reading taken. This was repeated until no further changes in fluorescence were observed.

Polyplex formation

Six micrograms of plasmid DNA was pipetted into 150 µl of 20 mM HEPES, pH 7.4 (final plasmid DNA concentration of 20 mg/ml). CTb-K₁₀₀ or K₁₀₀ at N : P of 0.6, 0.9, 1.2, 1.5, 2.0 or 3.8 was diluted into 150 µl of

20 mM HEPES, pH 7.4. The CTb-K₁₀₀ or K₁₀₀ solution was pipetted into the DNA solution and immediately mixed by vortexing. Polyplexes were left to form for 60 min at room temperature prior to use.

Cell culture

Rat PC12 pheochromocytoma cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated horse serum (Life Technologies), 5% foetal bovine serum (FBS) and 2 mM L-glutamine. Cells were seeded at a density of 10⁵/ml on rat tail collagen type 1 (Collaborative Biomedical Products, Oregon, USA) coated tissue culture dishes at a concentration of 150 µg/ml. Differentiated PC12 cells were obtained by plating normal cells at the same cell density of 10⁵ cells/ml, with the growth media supplemented with 2.5s mouse NGF (Caltech Biosystems, Towcester, UK) at 50 ng/ml for 14 days. Cells were maintained in incubator at 37°C in 5% CO₂, water-saturated atmosphere.

Transfections

Cells were plated at the required density (96-well plate: 25 000 cells/ml; 24 well plate: 10⁵ cells/ml). Transfections were carried out with 2 µg of plasmid DNA per well and left for 5 h at 37°C. After 5 h the transfection media was removed and appropriate fresh media added (either normal or NGF supplemented). Cells were assayed for transgene expression 48 h post-transfection.

Competition of CTb and CTb-K₁₀₀

Normal or NGF-differentiated PC12 cells were incubated for 30 min with 100-fold excess of free CTb (Calbiochem) prior to the addition of 6 µg of pCMV-DNA_{LacZ} condensed with either CTb-K₁₀₀ or K₁₀₀. After 5 h, the transfection media was removed, replaced with appropriate fresh media and the cells assayed 48 h post-transfection for β-gal activity.

β-Galactosidase assay

Total β-gal activity was quantified using a chemiluminescent reporter assay system Galacto-Light Plus™ (Tropix, Cambridge, UK). Cells were lysed in 200 µl of lysis solution (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol). Then, 50 µl of the lysate were added to 200 µl of chemiluminescent substrate solution (Galacton-Plus in 100 mM sodium phosphate, pH 8.0, 1 µM magnesium chloride.) After incubation for 1 h, 300 µl of luminescence accelerator reagent were added and the sample assayed on a monolight Lumat LB9501 luminometer (Berthold, UK) by counting emitted light

units for 10 s. Transfection was quantified as β-gal activity measured per mg of protein (relative light units/mg, RLU/mg), where cell protein concentration was calculated using the BCA assay (Sigma, Poole, UK).

BCA assay

The BCA protein assay (Sigma, Poole, UK) was carried out on 50 ml of cell lysis solution according to the supplied protocol.

FACS analysis

Analysis of GFP expression in PC12 cells was performed using a Coulter-Epics XL flow cytometer. Cells were harvested 48 h post-transfection by trypsinisation, washed with PBS, and then fixed in 4% paraformaldehyde. GFP fluorescence was analysed using an argon-ion laser tuned to 488 nm, with emitted light collected with dichroic sets of 520 and 575 nm to enable the removal of autofluorescence by diagonal gating [23]. Background and autofluorescence was determined using cells treated with empty plasmid. Cellular debris showing reduced lateral and orthogonal scatter was excluded from analysis. The software package WinMDI ver 2.8 [24] was used to analyse data, which was expressed as the percentage GFP-positive PC12 cells.

Atomic force microscopy

Samples containing complexes in water (2 µl) with a final DNA concentration of 20 µg/ml were deposited onto the centre of a freshly split mica disk (Agar Scientific, Essex, UK). Following adsorption for 1–2 min at room temperature, excess fluid was taken off by absorption onto filter paper. The mica surface was dried at room temperature before imaging using an AFM-2, part of the Nanoscope II system (Digital Instruments). A Si₃N₄ cantilever with a spring constant of 0.12 N/m and a D scanner head were used. The scanning speed was 4.34 Hz.

Results

Physicochemical characterisation of CTb-K₁₀₀ polyplexes

The formation of CTb-K₁₀₀ and K₁₀₀ polyplexes was followed fluorometrically using an ethidium bromide exclusion assay. The electrostatic collapse of plasmid DNA excluded ethidium bromide from the major groove of the plasmid DNA, and, as a result, the formation of polyplexes was seen as an associated reduction in fluorescence. By adding either CTb-K₁₀₀, or K₁₀₀ in

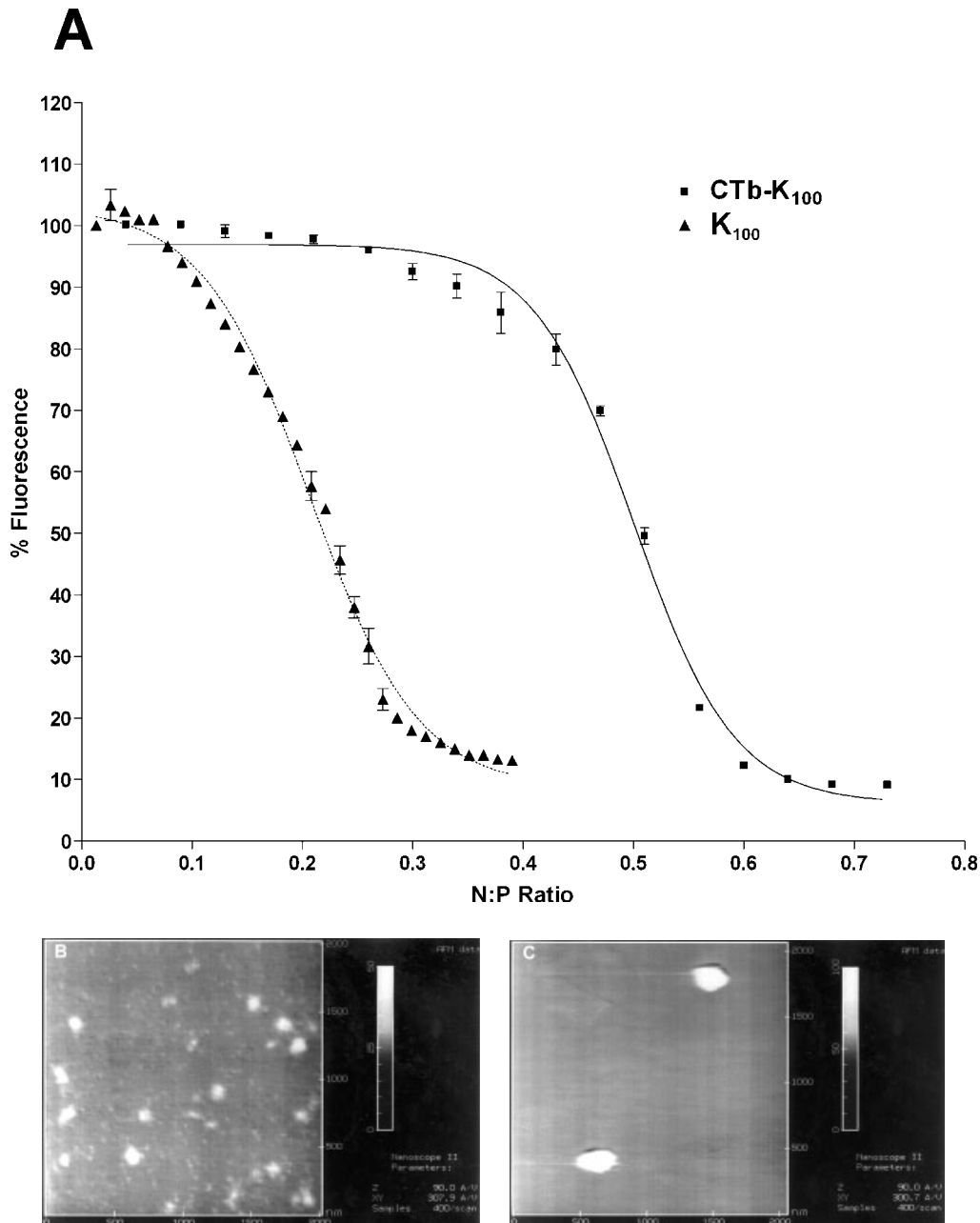


Figure 1. Physicochemical characterisation of CTb-K₁₀₀ and K₁₀₀ polyplexes. (A) Ethidium bromide condensation curve of plasmid DNA and polycationic polymers. Condensation of plasmid DNA by polycations was determined using plasmid DNA (20 mg/ml). Further, CTb-K₁₀₀ or K₁₀₀ added in 0.013 N:P aliquots were mixed and then measured. This was repeated until no further changes in mean fluorescence were observed (\pm SD, $n = 6$). Physical size of polyplexes was determined using AFM. Polyplexes at a plasmid DNA concentration of 20 μ g/ml and an N:P ratio of 2.0 were formed using either (B) K₁₀₀ or (C) CTb-K₁₀₀ polycations, and plated onto a mica surface. The results are representative of three different experiments

step-wise aliquots equivalent to an N:P ratio of 0.013, the extent of condensation was assessed. Figure 1A shows that at an N:P ratio of 0.65, the CTb-K₁₀₀ polyplexes are fully condensed ($n = 3$). The addition of further CTb-K₁₀₀ to the pCMV-DNA_{LacZ} solution resulted in no further reduction in fluorescence (data not shown). By comparison, using K₁₀₀, the pCMV-DNA_{LacZ} was fully condensed at an N:P of 0.32 ($n = 3$; Figure 1A), suggesting a role for the CTb protein in modulating the collapse of the plasmid DNA. Atomic force microscopy (AFM) was used to determine the

morphology and physical size of CTb-K₁₀₀ and K₁₀₀ polyplexes at the optimum N:P ratio/DNA concentration of 2.0/20 μ g/ml, for transfection, as determined by *in vitro* transfection rates (Figure 2A). Figure 1B shows pCMV-DNA_{LacZ} condensed with K₁₀₀. These polyplexes displayed a heterogeneous distribution of sizes, with an average diameter of 50 nm (Figure 1B), and are similar to those reported previously [25]. CTb-K₁₀₀ polyplexes were larger than those seen with K₁₀₀, revealing an average diameter of 150 nm, and were homogeneous in size (Figure 1C).

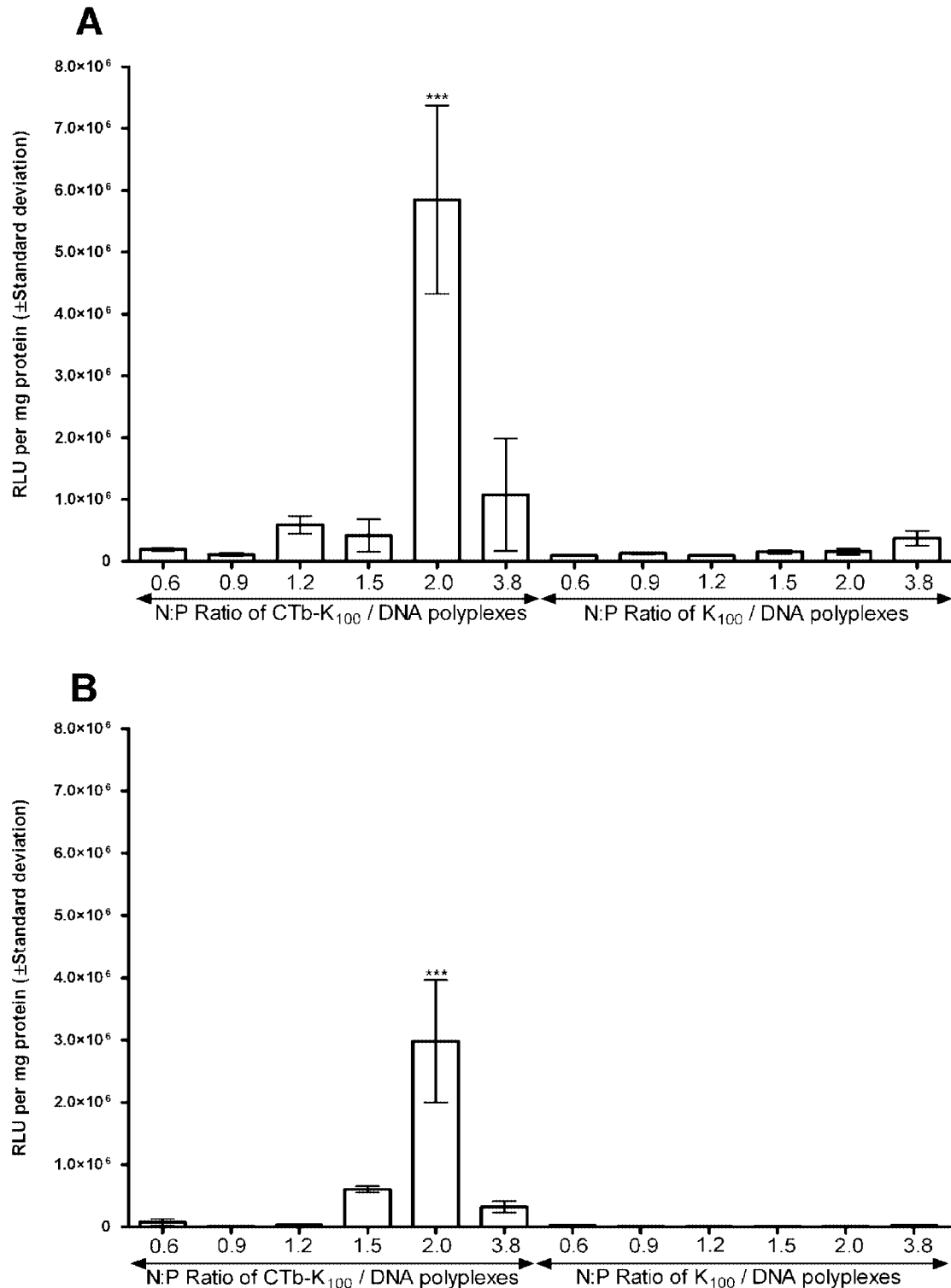


Figure 2. Optimisation of transfection of undifferentiated and NGF-differentiated PC12 cells with pCMV-DNA_{LacZ}/CTb-K₁₀₀ polyplexes. Polyplexes were formed at N:P ratios of 0.6, 0.9, 1.2, 1.5, 2.0 and 3.8 with either CTb-K₁₀₀ or K₁₀₀ (see Materials and methods). These polyplexes were applied to either (A) undifferentiated PC12 or (B) NGF-differentiated PC12 cells for 5 h, and the level of gene expression assayed 48 h post-transfection. The optimal N:P ratio was 2.0, with the level of gene expression falling in differentiated PC12 cells but the optimum N:P remaining at 2.0. Expression is shown as \pm SD ($n = 6$). ANOVA with Bonferroni post-test analysis revealed $***p < 0.001$, comparing CTb-K₁₀₀ N:P 2.0 with K₁₀₀ N:P 2.0 in both undifferentiated and NGF-differentiated PC12 cells

In vitro transfection optimisation of polyplex N : P

By applying pCMV-DNA_{LacZ} condensed with CTb-K₁₀₀ or K₁₀₀ at increasing N:P ratios over a broad range (0.6–3.8), the optimal cationic ratio for transfection with CTb-K₁₀₀ was determined in both undifferentiated (Figure 2A) and NGF-differentiated PC12 cells (Figure 2B). Transfection of undifferentiated PC12 cells with CTb-K₁₀₀ condensed cDNA encoding β -gal (Figure 2A) at N:P of 0.6 (evidenced by reporter gene expression levels of 0.19×10^6 relative light units (RLU)/mg protein; $p > 0.05$), 0.9 (0.11×10^6 RLU/mg protein; $p > 0.05$), 1.2 (0.59×10^6 RLU/mg protein; $p > 0.05$) and 1.5 (0.49×10^6 RLU/mg protein; $p > 0.05$) exhibited statistically lower levels of transfection compared with the maximum value of 5.84×10^6 RLU/mg protein at an N:P of 2.0 ($p < 0.001$; Figure 2A). The expression levels fell to 1.07×10^6 RLU/mg protein at the higher N:P of 3.8 (1.07×10^6 RLU/mg protein; $p > 0.05$). By contrast, transgene expression levels seen at the optimal N:P ratio with the unconjugated polycation, K₁₀₀, was lower than that seen with CTb-K₁₀₀, with the CTb-K₁₀₀ N:P of 2.0 giving nearly 30 times more LacZ expression than that achieved with the equivalent formulation of K₁₀₀ ($p < 0.001$; Figure 2A).

We next investigated the levels of transgene expression achieved with these polyplexes in NGF-differentiated PC12 cells (Figure 2B). By adding 50 ng/ml of NGF for 14 days, the PC12 cells were changed from a mitotic phenotype to a differentiated, post-mitotic neuronal-like phenotype [26,27]. Transfection of NGF-differentiated PC12 cells resulted in a lower but significant level of pCMV-DNA_{LacZ} reporter gene expression from CTb-K₁₀₀ polyplexes at all N:P ratios when compared with non-differentiated PC12 cells (5.84×10^6 RLU/mg protein in the undifferentiated cells, Figure 2A, and 2.98×10^6 RLU/mg protein in the NGF-differentiated cells, $p < 0.001$; Figure 2B). The maximal level of transgene expression was still observed at an N:P ratio of 2.0. By contrast, in differentiated PC12 cells, no detectable transgene expression was seen with K₁₀₀ polyplexes at any N:P ratio (Figure 2B).

Frequency of transfection

Undifferentiated cells were transfected at a range of N:P ratios using a pCMV-DNA_{GFP} reporter gene construct to reveal the percentage of successfully transduced PC12 cells. By this analysis the optimal CTb-K₁₀₀ N:P ratio for transfection remained at 2.0, with FACS analysis showing a level of 7.36% of undifferentiated PC12 cells expressing GFP at 48 h post-transfection ($p < 0.001$ vs. K₁₀₀ N:P 2.0; Figures 3A and 3C). This was significantly higher than the highest level of 0.36% seen with K₁₀₀ ($p < 0.001$; Figure 3C). Differentiation of PC12 cells with NGF did not alter the optimal N:P of 2.0 at which the maximum pCMV-DNA_{GFP} expression was seen with

the CTb-K₁₀₀ polyplexes, and the percentage of cells expressing GFP achieved at this N:P was still 2.17% ($p < 0.001$; Figures 3B and 3D). This was in stark contrast to 0% transfection with the untargeted polyplexes in both differentiated and undifferentiated PC12 cells.

Competitive binding of GM1

An excess of 100-fold free CTb was used as a competitive inhibitor for CTb-K₁₀₀ transfection. The free CTb was preincubated with either undifferentiated or differentiated PC12 cells at a concentration of 100 μ g/ml for 30 min before the addition of the CTb-K₁₀₀ polyplexes. This was repeated for K₁₀₀ as a control. Transfection of undifferentiated PC12 cells with CTb-K₁₀₀ at the optimum determined N:P of 2.0 produced a 15-fold higher transgene expression than K₁₀₀ (compare A + E bars in Figure 4). However, the level of transfection was reduced from 6.72×10^6 RLU/mg protein to 0.5×10^6 RLU/mg protein when the transfection was conducted in the presence of free CTb ($p < 0.001$; compare bars A + B in Figure 4), suggesting that the binding and uptake of CTb-K₁₀₀ polyplexes was specific and mediated through GM1. Similarly, CTb-K₁₀₀ transfection of NGF-differentiated PC12 cells could be competitively inhibited with free CTb, from 1.97×10^6 RLU/mg protein to 0.57×10^6 RLU/mg protein ($p < 0.01$; compare bars C + D in Figure 4). There was no statistically significant alteration in the low levels of transgene expression from K₁₀₀ polyplexes after the addition of free CTb in either undifferentiated or NGF-differentiated PC12 cells ($p > 0.05$; compare bars E–H in Figure 4).

Discussion

The development of novel targeted non-viral cationic polymers could provide an opportunity for a safe, well-defined, efficient therapeutic gene delivery system for the treatment of CNS injury following trauma. A number of targeting ligands have been employed previously to aid gene delivery in the CNS, including the H_c fragment of tetanus toxin [9] and neurotensin [28]. Whilst these ligands improved uptake and expression of reporter genes by 10- and 6.5-fold, respectively, in dividing neural cell lines, neither has been evaluated in post-mitotic neurons where transfection rates would be significantly reduced. However, the encouraging results obtained with these ligands have prompted the evaluation of CTb as a neuronal gene targeting ligand. Using CTb, which specifically binds to the GM1 ganglioside, we hoped to improve the transfection efficiency in the adult CNS. The GM1 ganglioside, which is a ubiquitous component expressed in both glia and neurons in the adult CNS [19], has a higher level of expression in neurons than that seen in glia [29,30]. Although there will be uptake and expression in glia due to the presence of GM1, we predict that the majority of polyplex uptake will be

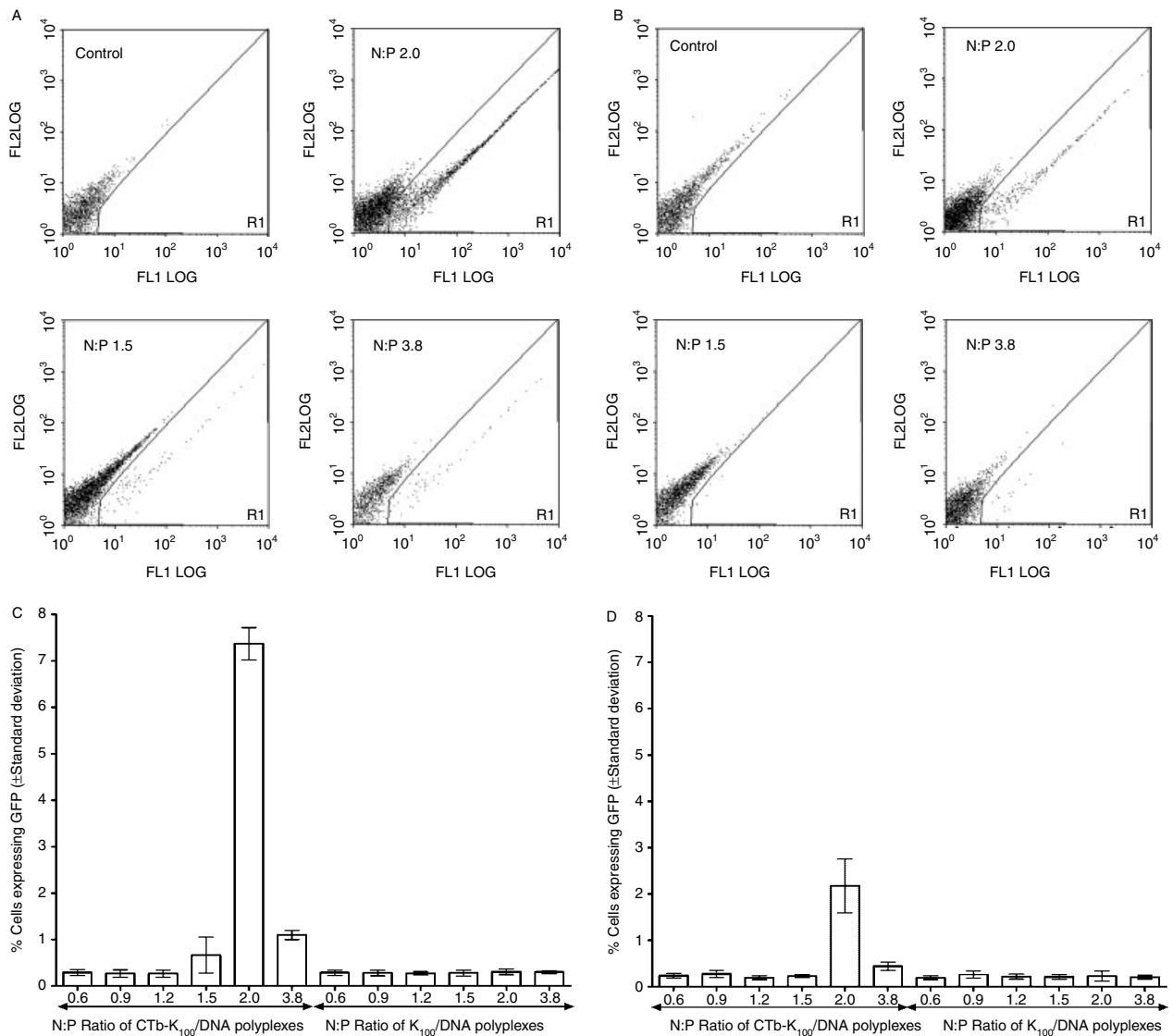


Figure 3. Frequency of transfection with pCMV-DNA_{GFP}/CTb-K₁₀₀ polyplexes in undifferentiated and NGF-differentiated PC12 cells. Polyplexes were formed at N:P ratios of 0.6, 0.9, 1.2, 1.5, 2.0 and 3.8 with either CTb-K₁₀₀ or K₁₀₀. These polyplexes were applied to either (A, C) undifferentiated PC12 or (B, D) NGF-differentiated PC12 cells for 5 h, and the level of gene expression assayed 48 h post-transfection using flow cytometry as described in Materials and methods. The solid diagonal line represents the demarcation point of fluorescent and non-fluorescent cells, as produced by gating the WinMDI software against a control of PC12 cells transfected with empty plasmid. Cells lying below the diagonal line in region R1 are cells that are expressing eGFP following transfection. In agreement with the optimal level of protein expression seen in Figure 2, the greatest number of cells expressing GFP was at the optimal N:P ratio of 2.0. The number of cells expressing GFP fell in differentiated cells but the optimum ratio remained at 2.0. Expression is shown as \pm SD ($n = 6$). ANOVA with Bonferroni post-test analysis revealed $***p < 0.001$, comparing CTb-K₁₀₀ N:P 2.0 with K₁₀₀ N:P 2.0 in both undifferentiated and NGF-differentiated PC12 cells

associated with neurons. GM1 is also expressed in the chosen neuronal paradigm, PC12 cells [31,32], with the distribution pattern of GM1 closely regulated by the cell cycle control of ganglioside synthesis [33].

The physicochemical characterisation of both CTb-K₁₀₀ and K₁₀₀ polyplexes revealed that the presence of the large 55-kDa CTb protein affected the kinetics of polyplex formation, as determined by ethidium bromide exclusion assay; however, the AFM analysis revealed that there was no flocculation of the CTb-K₁₀₀ polyplexes. The presence of large hydrophobic proteins is reported to affect the size of polyplexes [23]; however, the CTb-K₁₀₀ polyplexes we have generated were a homogenous

population of relatively small, well-defined particles. This was in contrast to the AFM of K₁₀₀ polyplexes which showed particles of different sizes, that could be due to the heterogeneous nature of the stock K₁₀₀ used for their formation. The K₁₀₀ used for conjugation to CTb was purified to give a homogenous preparation of lysine. The difference in the size of the CTb-K₁₀₀ and K₁₀₀ particles was thought to have a negligible effect on transfection of PC12 cells, with any effect seen, we anticipate, due to the presence of receptor-mediated uptake via CTb. Previous studies by several groups using cationic liposomes [34] and PEI [23], in cultured neuronal cell lines, have shown that only very large particles, >500 nm, start to exhibit an

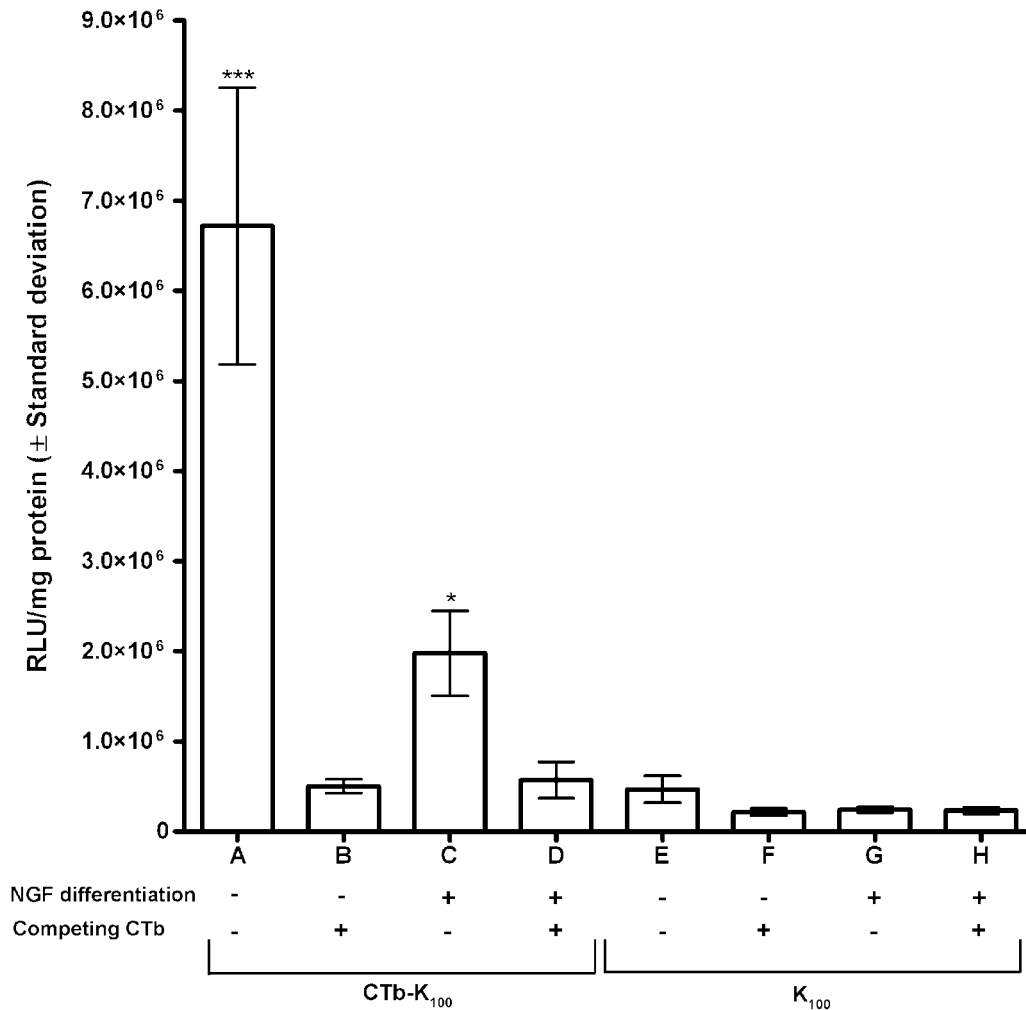


Figure 4. Competitive inhibition of pCMV-DNA_{LacZ}/CTb-K₁₀₀-mediated transfection using free CTb. To investigate whether the transfection of undifferentiated and NGF-differentiated PC12 cells with CTb-K₁₀₀ polyplexes was mediated through GM1 binding, we investigated the competitive inhibition with free CTb. (A) CTb-K₁₀₀/DNA_{GFP} undifferentiated cells; (B) CTb-K₁₀₀/DNA_{GFP} + 100 µg/ml CTb undifferentiated cells; (C) CTb-K₁₀₀/DNA_{GFP} NGF-differentiated cells; (D) CTb-K₁₀₀/DNA_{GFP} + 100 µg/ml CTb NGF-differentiated cells; (E) K₁₀₀/DNA_{GFP} undifferentiated cells; (F) K₁₀₀/DNA_{GFP} + 100 µg/ml CTb undifferentiated cells; (G) K₁₀₀/DNA_{GFP} NGF-differentiated cells; and (H) K₁₀₀/DNA_{GFP} + 100 µg/ml CTb NGF differentiated cells. Expression is shown as ±SD (*n* = 6). ANOVA with Bonferroni post-test analysis revealed ****p* < 0.001, comparing CTb-K₁₀₀ N : P 2.0 in undifferentiated PC12 cells, and **p* < 0.01, comparing CTb-K₁₀₀ N : P 2.0 in NGF-differentiated PC12 cells

enhanced transfection, presumably by a combination of two effects, firstly, sedimentation of denser particles onto the cell surface, and, secondly, the effect of these large particles on the bursting of endosomes once internalised into the cell.

For transfection analyses we used rat PC12 cells as these cells grow normally in defined growth media, but upon the addition of 50 ng/ml of NGF for 14 days the cells stop dividing, throw out neurite processes and resemble a neuronal phenotype [26,27,35]. Using undifferentiated PC12 cells, the optimal N:P ratio for transfection with both CTb-K₁₀₀ and K₁₀₀ particles was shown to be 2.0, with a 36-fold increase in expression levels and a 20-fold increase in the frequency of transfection, with the expression levels falling off significantly at higher ratios. The observed decrease in expression at higher N:P ratios could be explained by the presence of excess unbound polycation which is normally present following polyplex

formation [36]. The increasing levels of free CTb-K₁₀₀, at N:P ratios above 2.0, could cause inhibition of polyplex binding to cells, by competing with CTb-K₁₀₀ polyplexes for GM1 binding sites. N:P ratios lower than 2.0 produced a transfection level that was very low and equivalent to that obtained with untargeted poly(D-lysine).

Importantly, CTb-K₁₀₀ enhanced transgene expression levels in both undifferentiated and NGF-differentiated PC12 cells, with an optimum N:P ratio of 2.0 in both. In differentiated PC12 cells, CTb-K₁₀₀ gave an 11-fold increase in the frequency of transduction compared with K₁₀₀ and a remarkable 133-fold enhancement of reporter gene expression levels compared with K₁₀₀. This compares favourably with the 10- and 6.5-fold increase in expression levels seen by others with H_c tetanus toxin-K₁₃₁ and neurotensin-K₂₁₄ in mitotic neuronal cell lines [9,28]. The lower levels of transgene expression observed in differentiated PC12 cells presumably reflects their

different mitotic state, shifted from cycling to G₀, i.e., to one resembling primary neurons, so that nuclear access of transcriptional machinery by the cDNA is reduced. The sequence of the cholera toxin B subunit [37], although not containing a classical NLS [38], does contain a number of basic residues which may facilitate the nuclear localisation of CTb-K₁₀₀ polyplexes. Our observation of significant transfection of arrested cells with non-viral gene transfer systems is in agreement with several other studies [39–41]. When the frequency of transfection was examined, the number of cells transduced was significantly higher with CTb-K₁₀₀ than with K₁₀₀, which appears to result in little or no expression in both undifferentiated or NGF-differentiated cells. However, as predicted, the frequency of transduction achieved with CTb-K₁₀₀ polyplexes was lower upon NGF differentiation of PC12 cells to a neuronal phenotype. Although the arrest of PC12 cells in G₁/G₀ does increase the levels of GM1 synthesis and display on the cell surface [42], and therefore we predict increase the amount of CTb-K₁₀₀ targeted polyplexes into the cell interior, the nuclear envelope still presents the most significant barrier to transgene expression once inside the cell [4]. Transfection activity of CTb-K₁₀₀ polyplexes was greatly reduced in the presence of free CTb, suggesting that the uptake is receptor-mediated and specific to GM1. Cytotoxicity, assessed by an MTS assay, was not observed with free CTb (data not shown).

In summary, we have shown that CTb coupled to poly(D-lysine) can significantly enhance non-viral reporter gene delivery to neurons, by targeting cDNA to GM1-expressing cells and exploiting receptor-mediated uptake. This ligand-targeted vector system appears to be a promising alternative to viral systems for neuronal gene therapy and could have applications not only in the field of CNS penetrant injuries, but also in the treatment of other neurodegenerative conditions.

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