The Demonstration of Polyethylenimine Mediated Gene Transfer into the Rodent Hypothalamus Results in Persistent Over-Expression and Phenotypic Change

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ABSTRACT

Polyethylenimine (PEI) has been proposed as a non-viral vector, and has been successfully used to transfer reporter genes into the central nervous system (CNS), kidneys, and lungs of adult mice. Neuropeptide Y (NPY) is a peptide expressed in the hypothalamus and is important in the regulation of body weight. Using PEI combined with stereotactic microinjection, we have successfully transferred cDNA-encoding NPY driven by the cytomegalovirus (CMV) promoter into the arcuate nucleus of adult male Wistar rats. Animals treated with NPY expressing plasmids (pNPY) gained more weight than the controls ($p<0.05$), with associated increases in food intake ($p<0.05$) and decreased brown adipose tissue activity, measured by Guanosine Diphosphate (GDP) binding to mitochondria, ($p<0.05$). In a separate study, hypothalamic slices from the rats treated with pNPY/PEI showed increased NPY release (pNPY 9.7 ± 0.3 fmol/l vs. control 8.3 ± 0.5 fmol/l, $p<0.05$, n = 3). These results suggest that PEI is an effective vector for gene transfer into the rodent brain and can increase the protein production sufficient to result a persistent phenotypic change. This technique offers the potential of a simple and effective method to manipulate gene expression localised to specific regions of the adult rodent brain. *Iran. Biomed. J. 8 (1): 33-39, 2004*

INTRODUCTION

An efficient, reliable, and safe method of gene transfer into the mammalian central nervous system (CNS) would provide a valuable method for the study of neurophysiology and a potential therapeutic approach to human disease. Non-viral gene transfer agents have a number of advantages over viral gene transfer systems. Unlike viral vectors, there are no concerns regarding production of an infectious agent or introduction of potentially immunogenic viral proteins. There are a number of potential non-viral vectors including viral coat proteins [1], liposomes [2] and cationic lipids [3]. Whilst, the use of these vectors is well established *in vitro* but their use *in vivo* has been limited by their low efficiency and the transience of transgene expression.

Among the non-viral vectors, polyethylenimine (PEI) appears particularly promising with significantly higher transfection rates *in vitro* when compared to a number of other non-viral gene transfer agents [4]. PEI is a cationic polymer with every third atom, an amino nitrogen, which is protonated at physiological pH. This results in an interaction between PEI and DNA in the presence of 5% glucose, which produces condensed DNA toroids of between 20 and 40 nm [5]. This level of protonation also confers a large buffering potential on PEI, which may protect the DNA from degradation in the lysosomes and facilitate its release from them, by osmotic swelling. The condensing capacity of the PEI may aid transport of the transgene across the nuclear membrane [4]. In addition, using PEI, there are no limitations on the size or type of DNA that can be transferred. PEI has been successfully used *in vivo* to transfect adult mammalian neurones with the luciferase reporter gene [6]. Recently, PEI has been used as PEI/DNA-P53 vector in an orthotopic bladder cancer model [7].

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Neuropeptide Y (NPY) is a 36-amino acid peptide originally isolated from porcine intestine. It is the most potent stimulator of feeding known when administered by intracerebroventricular (ICV) injection [8]. NPY increases food intake through the hypothalamic NPY receptors especially the Y1 receptor [9-11]. In the hypothalamus, NPY is synthesised in the arcuate nucleus and has an important role in the regulation of body weight [12]. Repeated ICV injections of NPY result in obesity associated with reduced brown adipose tissue activity and increased food intake [13]. In addition to NPY that is the most potent stimulator of food intake, other peptides such as orexin-A and galanin also affect on food intake [14].

The aim of this study was to demonstrate the PEI mediated gene transfer into the hypothalamus to over-express NPY and determine the phenotypic changes.

MATERIALS AND METHODS

Total RNA was isolated from adult male rat hypothalamus using the acid guanadinium thiocyanate/phenol/chloroform extraction. Forty micrograms of the total RNA were reverse transcribed using avian myoblastoma virus reverse transcriptase in a reaction primed using oligo $dT_{(17)}$. Half of the resulting DNA was amplified by PCR using primers corresponding to nucleotides 2-22 and 420-440 (Table 1) of the NPY cDNA (g205756, Oswel DNA Services, UK). The reaction was cycled 25 times using the following conditions: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds. The product of the PCR reaction was cut using Hind III and Bam HI and inserted into pCDNA1.1$_{(amp)}$ (Invitrogen, Leek, the Netherlands), to produce pNPY.

Table 1. The sequence and position of oligo-nucleotides used in the production of NPY by PCR, along with the restriction enzymes.

Position of NPY cDNA	Sequence	Restriction site
2-22	AAGCTCATTCCTCGCAGAGGC	Hind III
420-440	TCATCCTGTGAAACCAGTCTG	Bam HI

Production of DNA/PEI complexes. The DNA-polyethylenimine (PEI) complex was produced by the method of Abdallah *et al.* [6]. Briefly, DNA at a concentration of 2 µg/µl in 5% (w/v) glucose was mixed with 6 equivalents of 0.1M PEI (average M_w

about 25,000, Aldrich, Poole, Dorset, UK) and vortexed for 30 seconds. The complexes were incubated at room temperature for ten minutes before use.

Stereotactic injections. Adult male Wistar rats about 300 g, (Harlan UK, Bicestor, Oxon, UK) were anaesthetised with 100 mg/kg ketamine and 20 mg/kg xylazine and immobilised in a stereotactic frame (David Kopf instruments, Tujunga, California, USA). An incision was made into the scalp and the dura mater was removed. A 0.65-mm burr hole was drilled into the skull, 3.8 mm caudal and 0.2 mm lateral to the bregma. A 23-gauge cannula was placed into the burr hole to a depth of 10 mm, coinciding with the medial portion of the mid-region of the arcuate nucleus and 1 µl of the DNA/PEI complex infused over one minute. The cannula was left in position for 10 minutes then slowly withdrawn. Animals were housed individually and maintained at 20°C with a twelve-hour light/dark cycle. All procedures were carried out in accordance with the UK Animal procedures (Scientific Act under UK Home office licence PPL 90'1077).

Chronic NPY over-expression study. Fifteen animals were randomised into two groups: one group received pNPY/PEI (n = 8), the other pcDNA1.1$_{(amp)}$/PEI (n = 7). After a five-day recovery period, food intake and body weight were recorded daily until the end of the study. All the controls and six of the pNPY treated animals were killed thirty-six days after the injection. The brown adipose tissue was removed for guanosine diphosphate (GDP) binding measurement.

Measurement of hypothalamic NPY release. Six animals were injected as described above with pNPY/PEI (n = 3), or pcDNA1.1$_{(amp)}$/PEI (n = 3). On day 7, the animals were killed and the brains removed for *in vitro* measurement of NPY release (see below).

GDP binding to brown adipose tissue mitochondria. Specific binding of GDP to brown adipose tissue mitochondria was quantified as a measure of brown adipose tissue activity using a slight adaptation of the method of Rial and Nicholls [15]. Mitochondria (0.3 mg/ml), prepared by the method of Saggerson and Carpenter [16], were incubated at 30°C for 5 min in a final volume of 250 µl of buffer B (100 mM KCL, 5 mM Tris-HCl pH 7.0, 2.5 µM rotenone, 25 µM GDP, 0.1 µCi [^{14}C] sucrose and 0.75 µCi [^{3}H] GDP) to give total

binding. Non-specific binding was measured in the additional presence of 1 mM GDP.

Association of GDP with the mitochondria was calculated after allowing for trapped extra-matrix volume, estimated by the $[^{14}C]$ sucrose space. Correction for non-specific binding was applied to all samples and results expressed as pmol bound/mg protein [16].

Measurement of NPY release from hypo-thalamus explants. The static incubation system used was a modification of the method of Beak *et al.* [17]. Male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with ventral surface uppermost and placed in a vibrating microtome (Bio-Rad Hemel Hempstead, Herts, UK). A 1.5-mm slice was taken from the base of the brain and immediately transferred into individual tubes containing 1 ml of artificial cerebrospinal fluid (aCSF) [20 mM $NaHCO_3$, 126 mM NaCl, 0.09 mM Na_2HPO_4, 6 mM KCl, 1.4 mM $CaCl_2$, 0.09 mM $MgSO_4$, 8 mM glucose, 18 mg/ml ascorbic acid and 100 µg/ml aprotinin, Trasylol (Bayer, Haywards Heath, UK)] equilibrated with 95% O_2 and 5% CO_2. The tubes were placed on a shaking platform in a water bath maintained at 37°C. After an initial 2-hour equilibration period, with aCSF replaced every 60 minutes, the hypothalamic were incubated for 45 minutes in 500 µl aCSF. The aCSF was removed and frozen at -20°C until the measurement of NPY by radioimmunoassay using reagents and methods developed in the laboratory that this work was carried out [18]. The viability of the tissue was verified by a 45-min exposure to 56 mM KCl; isotonicity was maintained by substituting K^+ for Na^+.

Western blotting. Western blotting was carried according to the method of Burnette [19]. Briefly, proteins were transferred on to Hybond-C (Amersham) using semi-dry transfer on a Multiphor II Bova Blot Electrophoretic transfer unit (Pharmacia LKB Biotechnology). After transfer, the nitrocellulose was rinsed in distilled water and incubated for 30 min. in TBS/Tween (1% Tween 20, 10 mM Tris, 0.9% NaCl pH 7.4) with 5% (w/v) non-fat dried milk to reduce non-specific binding. Blots were then incubated with a 1 µg/µl dilution of: rabbit anti-mouse uncoupling protein-1 (UCP-1), (Research Diagnostics, Inc., Flanders, NJ, USA) in TBE/Tween 20 for 90 min with gentle agitation. After hybridization to the primary antibody, filters were washed with TBS/Tween. The filters were incubated for one hour with a 1:4000 dilution of anti rabbit IgG conjugated to horseradish peroxidase in TBS/Tween. Bands were visualized using enhanced chemi-luminescence (ECL) and exposed to photographic film.

Statistical analysis. All results are given as the mean ± SEM. Only those animals that showed exogenous NPY expression correctly targeted to the mid-region of the arcuate nucleus were included in the statistical analysis. Results were analysed using a student's unpaired *t*-test. Significance was taken as *p*<0.05.

RESULTS

Weight gain and food intake. There was no significant difference in the mean body weights between the two groups at the start of the experimental period (304.1 ± 2.6 g vs. 295 ± 2.6 g; *p* = 0.5). Cumulative weight gain was significantly different between the groups by day 10 (pNPY 18.6 ± 1.3 g vs. control 12.3 ± 1.3 g, n = 7 *p*<0.01). This increase remained significant for the duration of the study (day 36: NPY 110 ± 4.0g vs. control 94 ± 3.1 g, *p*<0.05) (Fig. 1). The cumulative food intake of the NPY-treated animals was significantly increased in the pNPY-treated group by day 12 (pNPY 223.9 ± 5.2 g vs. control 208 ± 6.6 g. n = 7, *p*<0.05), (Fig. 2A). This change remained significant for the rest of the study (day 36: pNPY 960±20g vs. control 902 ± 23 g *p*< 0.05) (Fig. 2B).
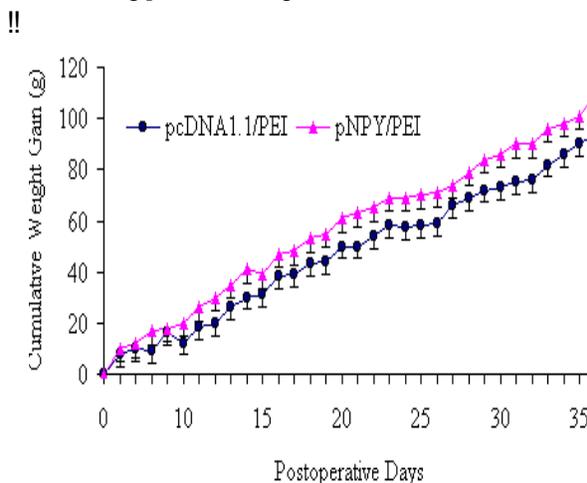

Fig. 1. Cumulative weight gain in rats following injection of 2 µg of either pCDNA$_{1.1amp}$ or pNPY complexed with 6 equivalents of 25 kDa PEI into the arcuate nucleus. Data represent mean ± SEM (n = 7) and were analysed using student's *t*- test.

Brown adipose tissue GDP binding. Mitochondrial binding of GDP was used as a measure of brown adipose tissue activity. This was significantly reduced in the pNPY group compared to controls (pNPY 17.5 ± 6.7 μmol/mg vs. control 45.7 ± 9.4 μmol/mg, *p*<0.05 n = 4), (Fig. 3).

NPY release in hypothalamic explants. NPY release from hypothalamic explants during the 45-minutes incubation was significantly greater in the pNPY than in controls (pNPY 9.7 ± 0.3 fmol/l vs. control 8.3 ± 0.5 fmol/1, *p*<0.05, n = 3), (Fig. 4).

Fig. 3. GDP binding to mitochondria isolated from brown adipose tissue removed from rats 36 days after injection of 2 μg of either pCDNA$_{1.1amp}$ (control) or pNPY. Data represent mean $\pm$ SEM (n = 4), *p*<0.05.

Western analysis of UCP-1 expression in brown adipose tissue. UCP-1 levels appeared to be reduced in the pNPY-treated animals (n = 4), (results not shown).
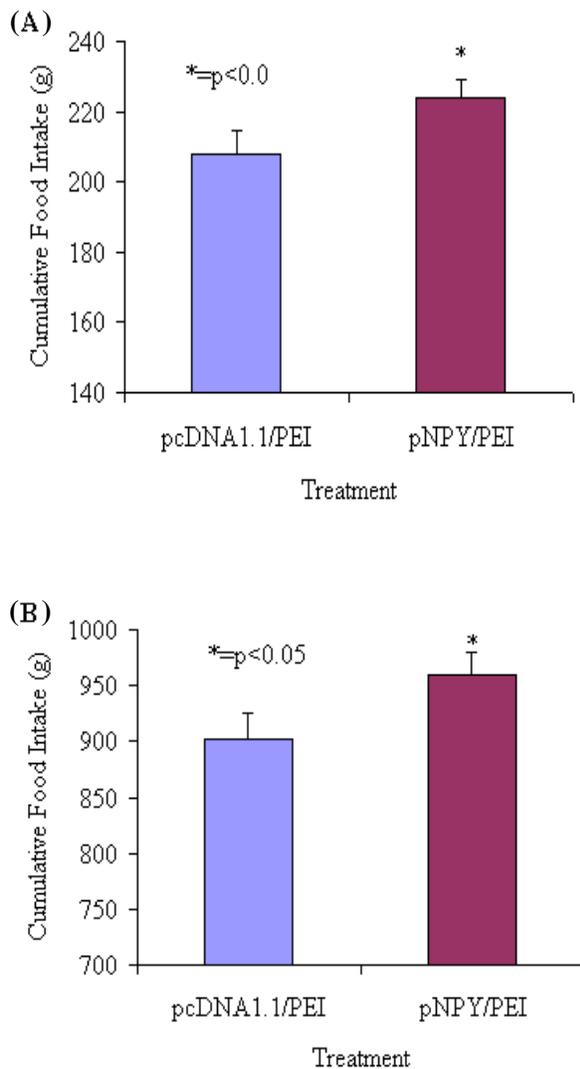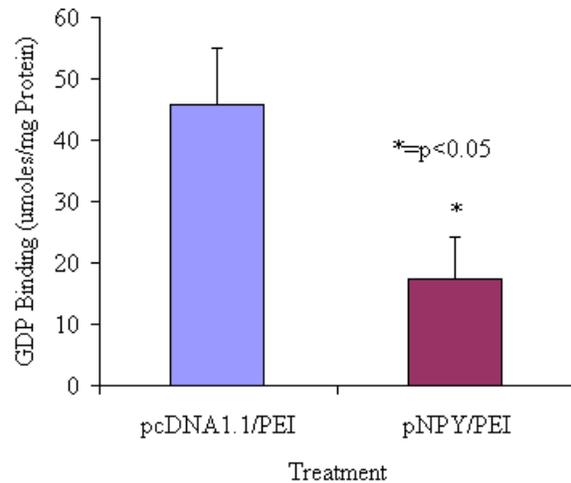
(A)

(B)

Fig. 2. Cumulative food intake of rats following injection of 2 μg of either pCDNA$_{1.1amp}$ or pNPY complexed with 6 equivalents of 25 kDa PEI into the arcuate nucleus. Data represent mean $\pm$ SEM (n = 7) and were analysed using student's *t*- test. **(A),** On day 12; **(B)**, On day 36.

DISCUSSION

In this paper, we report the successful use of PEI as a gene transfer agent into the arcuate nucleus of the adult rat brain. Using NPY as the model transgene, we obtained a significantly increased weight gain and food intake and a reduction in brown adipose tissue activity. In a separate study, we have also demonstrated that hypothalamus from animals treated with pPNY/PEI release more NPY than controls.

There are several candidates for use as non-viral gene transfer agents including liposomes, cationic polymers and viral proteins. All of them have been used with some degree of success either *in vitro* or *in vivo* often to deliver reporter genes. Of these candidates, PEI has shown particular promise as a gene transfer agent both *in vitro* and *in vivo*. Several researchers have reported the successful use of PEI *in vivo* using reporter genes, in the central nervous system (CNS), kidneys and lungs [20, 21].

Our data demonstrated that the use of PEI as a vector resulted in levels of transgene expression sufficient to cause a phenotypic change and these levels of expression could be maintained for at least 36 days. NPY release and expression have been
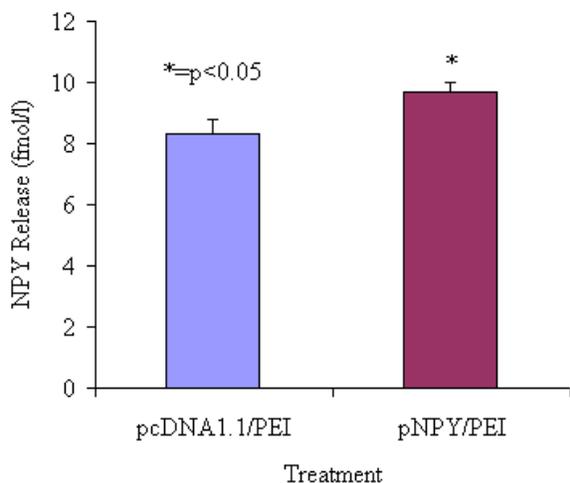
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Fig. 4. Release of NPY from hypothalamic slices maintained *in vitro* for two hours. Hypothalamus was dissected from rats seven days after injection of 2 μg of either pCDNA$_{1.1amp}$ (control) or pNPY. Data represent mean ± SEM (n = 3), $p<0.05$.

measured directly by hypothalamic explants and *in situ* hybridisation (data not shown). Expression has also been measured indirectly by the effects on weight gain, food intake and brown adipose tissue activity. In the second part of our study, we demonstrated a 125% increase in NPY secretion from hypothalamic slices from animals killed seven days after injection with pNPY. Taken together theses data argue strongly that PEI can be used to deliver genes to differentiated neurones with sufficient efficiency to produce increased protein secretion to drive a phenotypic change and that expression of the transgene can be maintained at these levels for considerable periods of time.

In this paper, we have not studied the time course of transgene expression in detail. The *in situ* hybridization performed on the two animals at days 42 and 56, respectively showed persistent, localized expression of NPY (results not shown). The body weight curves in Figure 1 continue to diverge up to the end of the study at day 36. The continued divergence of the body weight curves and *in situ* hybridization data together suggest that levels of expression are maintained beyond day 36. This is much longer than that of reported for the kidney where the majority of expression was lost after 14 days [20] and that of other reports in the CNS where levels of expression rapidly fell after four days [6]. There are several possible explanations for these differences between our results and those of others using PEI. The reporter genes could be producing an immune response targeted to cells expressing the

transgene and this could result in apparent loss of transgene expression. Alternatively, since the operation requires a 5-day recovery period before feeding, behaviour can be reliably studied. It is possible that expression has reached the plateau by the time data were collected from these animals.

Non-viral vectors are considered to be less effective than viral vectors. Whilst we have no direct comparison between the efficiency of this system compared to that of viral delivery, an indirect comparison can be made. NPY has been used as a transgene in a previous paper using adeno-associated virus (AAV) as the vector [22]. In this study, transgene expression was demonstrated indirectly by measurement of food intake and weight gain. NPY mRNA levels were not measured. In animals treated with AAV expressing NPY, the increase in weight was greatest at day 20 when the NPY transgene treated animals had gained approximately 233% more weight than controls [22]. In our study, the weight gain in the pNPY treated animals was greatest at day 10 when the pNPY treated animals had gained 151% more weight than the controls. The phenotypic response to NPY over-expression will be influenced by factors such as the strain and age of animal used. The lower efficiency of PEI may be an advantage in the study of CNS physiology where extremely high levels of expression are not required and may in fact be undesirable. In circumstances where higher levels of expression are required, these may be achievable with techniques successfully applied *in vitro* e.g. complexing with liposomes and covalent attachment of targeting ligands to PEI.

Our results suggest that PEI provides a robust system for *in vivo* gene transfer. All of the treated animals were positive when examined by *in situ* hybridisation (results not shown). The only animal in the pNPY group which failed to gain more weight and eat more food than the control animals was one in which NPY expression was confined to the sub-thalamic region of the brain, a region in which NPY has no function in the regulation of body weight and food intake. We have not identified the cell types that are expressing the NPY transgene but our data suggest that at least some of them are neuroendocrine and presumably neuronal. Neuropeptide precursor processing is a specialised function of neuroendocrine cells and unprocessed NPY is inactive. The alterations we obtained in food intake, weight gain and brown adipose tissue (BAT) activity suggest that the NPY is being correctly processed. Additionally, the RIA used to quantify the NPY secreted by the hypothalamic slices is unable to detect unprocessed NPY (Fig. 4).

Whilst the use of PEI will probably not replace the use of viral vector systems, for many applications it does offer a number of advantages. This is particularly true in the study of CNS physiology where extremely high levels of expression are not required and may in fact be undesirable. The use of PEI as a vector could also prove valuable in the identification of targets for gene therapy in the CNS before the more arduous task of producing recombinant virions is undertaken. It is possible to chemically modify PEI relatively easily, for example by addition of cell targeting ligands [23] or nuclear localisation signals [24] and this might increase the efficiency of this system still further. It may also be possible to boost expression levels system further by alterations to the promoter enhancer elements used in the construct.

In conclusion, our data demonstrate that it is possible to use unmodified PEI to transfer genes into the adult rodent CNS resulting in increased levels of hormone secretion sufficient to produce a phenotypic change sustained for at least 36 days.

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