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# **Evaluation of Intranasal Gene Delivery by a Lipoplex Formulation**

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## **Abstract**

**Parkinson's disease (PD) is a debilitating neurodegenerative disorder that results in the progressive loss of dopaminergic (DA) neurons from a midbrain nucleus known as the substantia nigra. Loss of these neurons, which project to the dorsal striatum, an area involved in initiation of voluntary movement, causes motor deficits that include resting tremor, bradykinesia, and rigidity. Available treatment options seek to replace dopamine, directly stimulate striatal neurons, or extend the lifetime of remaining dopamine but such treatments do not prevent disease progression and all are subject to decreasing efficacy over the course of long-term use. Glial cell-line derived neurotrophic factor (GDNF) is a neuroprotective protein that has emerged as a therapeutic agent with the potential to halt the loss of dopaminergic neurons caused by PD. However, GDNF must reach structures deep within the brain to be effective, a problem that currently necessitates invasive surgery to deliver the protein. Gene therapy is one method for achieving long-term expression of therapeutic proteins, but available methods for delivering genes rely on viral vectors that can be both immuno- and oncogenic. With this study, we sought to assess a non-invasive, non-viral method for delivering therapeutic genes to the rat brain. Lipoplex plasmid DNA vectors encoding EGFP were administered intranasally to bypass the blood brain barrier. Analysis via fluorescence microscopy and sandwich ELISA revealed no significant differences in the level or apparent EGFP between animals that received empty liposomes, naked pEGFP, and pEGFP lipoplexes. However, several confounding variables were encountered and further study is warranted.**

## **Introduction**

### *Parkinson's Disease*

Parkinson's disease (PD) is a progressive, neurodegenerative disease which causes impaired motor function in approximately 1 million people in the United States [1] and 2% of the population over the age of 60 [2]. The primary symptoms of PD are resting tremor, bradykinesia, and rigidity although postural instability and akinesia are also common [1-3]. Furthermore, PD can result in fatigue, impaired cognitive ability, disturbances in sleep and autonomic function, and depression [3]. Together, these symptoms severely impede the quality of life for PD patients.

Although the etiology of PD is currently unknown [1, 3], it is well characterized by distinct pathological findings: namely, the loss of dopaminergic neurons in a midbrain nucleus known as the substantia nigra pars compacta (SNc) [1-4]. Normally, projections from the SNc release dopamine (DA) in the dorsal striatum (dST) to allow voluntary movement through the action of two distinct motor pathways. The direct striatonigral pathway, involved in the initiation of movement, is excited by DA while the indirect striatopallidal pathway, involved in the suppression of movement, is inhibited by DA interaction [2, 4-5]. Thus, the net effect of DA neuron loss observed in PD patients is excessive suppression of movement [4].

### *Treatment of Parkinson's Disease*

Conventional pharmacological treatments for Parkinson's disease (PD) aim to replace dopamine (DA) in the nigrostriatal pathway, directly stimulate striatal DA receptors, or extend the lifetime of DA released by surviving nigrostriatal DA neurons [6-

8]. These effects are accomplished by levodopa, DA agonists, and catechol-O-methyl transferase (COMT) and monoamine oxidase-B (MAO-B) inhibitors respectively. Levodopa, which is converted to DA by the enzyme aromatic L-amino acid decarboxylase (AADC), is the most widely-used and effective treatment for the majority of PD symptoms; however, treatment with levodopa is less effective for PD symptoms such as postural instability and can result in dyskinesia [7]. Compared to levodopa, long-acting DA agonists (e.g. bromocriptine, ropinirole, and pramipexole) may remain active for an extended duration with less propensity for inducing dyskinesia [7] and can improve all PD symptoms but they are typically less effective, more expensive [6], and are more likely to cause peripheral side effects [8]. Conversely, MAO-B and COMT inhibitors increase endogenous levels of DA by inhibiting enzymes involved in DA and levodopa metabolism, respectively [9]. While current pharmaceutical therapies can alleviate early PD symptoms, all are subject to decreasing efficacy over the course of treatment and do not prevent disease progression [7-8].

Since its discovery in 1993, an increasing body of evidence has shown that glial cell-line derived neurotrophic factor (GDNF) both protects and stimulates fiber outgrowth of dopaminergic neurons in rodent and primate models of PD [1, 7, 10-12]. Furthermore, a 2008 study performed by Alberto Pascual and others suggested that GDNF is essential for survival of all catecholaminergic neurons in adult mice: particularly DA neurons in the substantia nigra (SN) and ventral tegmental area (VTA) as well as noradrenergic neurons in the locus coeruleus [10]. Such observations have made GDNF a viable therapeutic candidate for halting the progressive loss of DA neurons that occurs

in the SNc of PD patients. So far, clinical trials appear to validate the use of GDNF for treating PD [7, 10]. However, issues regarding the method of administration [7] must be addressed before GDNF is considered a viable treatment option.

### Gene Therapy

The use of gene therapy for the treatment of human disease has been an attractive prospect over the last few decades [13-14] and several studies have successfully achieved expression of therapeutic proteins [13-16]. A clinical trial conducted in 2000 demonstrated gene therapy's ultimate potential in the case of X-linked severe combined immunodeficiency syndrome (SCID-X1); nine children from the study have shown long-term improvements in their symptoms [17] and three are purported to have been cured of the otherwise fatal immunodeficiency disorder [16]. However, the success of gene therapy has been largely hampered by its reliance on viral vectors as a means of delivering genes [14-15, 17-19]. While viruses have evolved molecular mechanisms which allow them to efficiently transfect cells [15-16], serious safety concerns have been raised with regards to their immunogenicity and oncogenicity [14, 16]. In 1999, a teenage participant in one clinical trial died from an adverse immune reaction to the adenovirus vector being used [15-16]. Several years later, two patients from the SCID-X1 trial developed T-cell leukemia as a result of the retroviral vector being inserted near the promoter sequence of a proto-oncogene [16-17]. Such setbacks have prompted the search for a safe and effective method to deliver genes without the use of viruses.

A potential alternative to viral gene therapy is the use of a non-viral vector for delivery of plasmid DNA encoding the gene of interest. During the 1980's, Jon Wolff demonstrated that direct injection of naked DNA, without use of a vector, into skeletal muscle tissue resulted in gene expression which lasted for several successive weeks [15, 19]. This simple method for delivering genes has produced similar results in liver tissue [15] and skin as well as particular types of tumors and immune cells [19]. However, little success has been achieved in other cell and tissue types [15]. First introduced in 1987 by Philip Felgner, cationic lipid vectors, namely cationic lipid/DNA complexes (lipoplexes), provide another possible alternative to viral vectors for the introduction of therapeutic genes into cells [13-15, 19]. It is believed that lipoplexes are not immunogenic [13], and they have proven safe when delivered locally at low doses [19]. Indeed, a number of clinical trials implementing lipoplex gene therapy for the treatment of cancer and cystic fibrosis appear to validate their use as effective, non-viral vectors [19].

### Intranasal Drug Delivery

The blood-brain barrier (BBB) has been a difficult obstacle in the successful delivery of drugs to the brain [20-27]. The specializations constituting the BBB serve to separate the chemical constituents of the systemic circulation from those in the brain [22-23] and is largely impermeable to hydrophilic compounds and molecules with a molecular weight (MW) above 500 da [24, 26]. As a result, neuroprotective proteins such as insulin-like growth factor-I (IGF-I; MW = 7.65 kDa), nerve growth factor (NGF; MW = 30 kDa), and GDNF (MW = 30 kDa) cannot cross the BBB and their use to treat diseases of the central nervous system (CNS) such as PD has necessitated direct

intracerebral infusions. Such an approach involves invasive surgery to target deep brain structures and carries a significant risk to patient safety [20, 22, 24-26]. Thus, finding an effective, non-invasive route for delivering large biomolecules to the brain has become an integral goal in the treatment of CNS diseases.

One promising alternative for delivering therapeutic proteins to the brain is the intranasal (IN) route of administration. This route bypasses the BBB providing a non-invasive approach to treating CNS diseases [20, 22, 24-25] that avoids the systemic circulation and associated peripheral side effects [20, 22]. The olfactory region located in the upper nasal passage possesses unique anatomy and physiology which is the source of this technique's viability [24, 27]. Following IN administration, substances appear to enter the brain primarily via paracellular transport across the nasal epithelium. Studies have shown that, once in the brain, these drugs primarily follow the tracts of the olfactory and trigeminal nerves [20, 24] whose terminal endings represent the only sensory neurons directly exposed to the external environment [24]. Successful IN delivery of both IGF-I and NGF has already been demonstrated in other laboratories [24-25] and with ovalbumin (OVAL; MW = 45 kDa) in the sponsor's laboratory [22]. These findings suggest that IN delivery of GDNF to the brain is also a feasible goal and potential method for treating PD.

### Purpose of Study

The primary goal of the current study was to assess the feasibility of IN plasmid DNA delivery using a lipoplex formulation as a non-invasive, non-viral means of delivering genes to the brain. As proof of principle, we sought to transfect cells with the

gene for enhanced green fluorescent protein (EGFP): a mutant version of green fluorescent protein (GFP; 26.9 kDa), naturally produced by the jellyfish *Aequorea victoria*, which has been optimized for brighter fluorescence. Due to their fluorescent properties, GFP and its derivatives, such as EGFP, are widely used tools for localization studies and supplies for quantitative assays are commercially available. Thus, our goals were to achieve uptake of lipoplexes and transfection of cells via IN administration, analyze where in the brain transfection had occurred using fluorescence microscopy, and determine the concentration of EGFP protein present in transfected areas using an enzyme-linked immunosorbent assay (ELISA). If successful, this study would support the use of this method to introduce therapeutic genes to the brain and inform future work in the sponsor's laboratory seeking to express GDNF in the brain as a means of protecting dopaminergic neurons in Parkinson's disease.

## **Methods**

### Experiment Design

A total of 80 male Sprague-Dawley rats (200-250g) were supplied by Taconic Farms, Incorporated (Hudson, NY). Animals were divided evenly into two analysis groups, one group for detection of EGFP expression by fluorescence microscopy (N = 40) and one for detection of EGFP expression by enzyme-linked immunosorbent assay (ELISA; N = 40). Both analysis groups were further subdivided into three treatment groups that received pEGFP lipoplexes, naked pEGFP, or plain liposomes (N = 24, 12, and 4 respectively).

### Preparation of Lipoplex and Liposome Formulations

One day prior to each IN delivery, dioleoylphosphatidylcholine (DOPC), cholesterol, and stearylamine, each in chloroform at a concentration of 10  $\mu\text{mol/mL}$ , were combined in a 50:30:5 ratio. The lipid mixture was divided into separate tubes for lipoplex and empty liposome batches, subjected to rotary evaporation for 30-60 minutes, and then freeze-dried for 2 hours to eliminate the chloroform. The resulting lipid film was stored overnight at 4  $^{\circ}\text{C}$  (with the exception of the 3<sup>rd</sup> preparation of 6 which was inadvertently left at room temperature; approximately 30  $^{\circ}\text{C}$ ). On the day of IN delivery, the lipids were reconstituted with either plasmid DNA or phosphate buffered saline (PBS). To create the lipoplexes, 50 parts of the EGFP expression vector (pEGFP-N1, Clontech Laboratories) was added at a concentration of 1  $\text{mg/mL}$  to the lipid film and the mixture was vortexed. To create the control liposomes, 50 parts PBS was added instead. Both control liposomes and lipoplexes were subjected to 4 alternating cold-hot cycles (on ice and in a water bath at approximately 37  $^{\circ}\text{C}$ , respectively) spending 2-10 minutes in each environment and vortexing between temperature transitions. The lipoplexes were formed by extrusion of the preparation using an Avanti Mini-Extruder with 100  $\mu$  filters. A 10  $\mu\text{L}$  sample was removed for particle size and zeta potential analysis using a Brookhaven Instruments Particle Size and Zeta Potential Analyzer.

### Intranasal Delivery

Rats were anesthetized with ketamine and xylazine (90/20  $\text{mg/kg}$ , i.p.) and placed in the supine position with their heads flat on the surface and noses upright (no

angle). Rats were given pEGFP lipoplexes, naked EGFP plasmid, or empty liposomes. Each preparation was administered using a Hamilton syringe fitted with a short piece of polyethylene tubing. Intranasal doses were given in 5  $\mu$ L increments every 4 minutes alternating nares for a total of 25  $\mu$ L per side (a total of 50  $\mu$ g of pEGFP DNA in rats given either lipoplexes or the naked plasmid). Rats remained supine for 60 minutes post-treatment.

#### *Detection of EGFP Expression by Fluorescence Microscopy*

Rats (2-6/group) were anesthetized with ketamine and xylazine (90/20 mg/kg, i.p.) and sacrificed by transcardial perfusion with 4% paraformaldehyde at 1, 3, 5 or 7 days after intranasal administration. Brains were post-fixed for 2 hours in 4% paraformaldehyde before being immersed in 30% sucrose for an additional 48-72 hours. Using a cryostat-microtome, 25-30  $\mu$ m thick coronal sections were cut, collected, and mounted on slides for observation under a fluorescent microscope (Olympus BX51 with a FITC excitation/emission filter set).

BIOQUANT image analysis software was used to count the number of green fluorescent cells in 15 sections taken from 5 representative regions along the rostral-caudal axis of each brain: frontal cortex (FC), striatum (ST), posterior forebrain (PFB), substantia nigra (SN), and posterior midbrain (PMB). Only cells whose fluorescence was clearly distinguishable above background were included in these counts.

#### *Detection of EGFP Expression by ELISA*

Rats (2-6/group) were anesthetized with isoflurane and sacrificed by rapid decapitation at 1, 3, 5 or 7 days after intranasal administration. The fresh brains were

cut to obtain sections containing regions of interest (i.e. FC, ST, PFB, SN, and PMB) and the sections were flash frozen in either liquid nitrogen (N<sub>2</sub>) or on dry ice (CO<sub>2</sub>) before being stored at -80 °C. Prior to the assay, each section was homogenized in 2 mL of lysis buffer (1% Igepal, 10% glycerol, and 1:100 protease inhibitor in PBS). Tissue homogenates were centrifuged at 4 °C for 30 minutes at 14,000 RPM. The supernatant was decanted and stored at -20 °C and pellets were discarded.

EGFP was detected by a sandwich ELISA developed in the sponsor's lab. For each ELISA, a 96-well plate was coated with 100 µL/well capture antibody (1:6000 in PBS; mouse anti-GFP Ab, Novus Biologicals, Cat # NB600-597) and incubated overnight at 4 °C. After this and each subsequent step, the plate was washed 3X with wash buffer (0.05% Tween-20 in PBS). Blocking buffer (5% sucrose in reagent diluent; 300 µL/well) was added and the plate was incubated at room temperature for 1 hour. The plate was then coated (100 µL/well) with EGFP standards (1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 pg/mL dilutions in reagent diluent (1% BSA in PBS), samples (supernatant), and blanks (reagent diluent) and incubated at room temperature for 2 hours. Next, the detection antibody (1:6000 in reagent diluent; rabbit anti-GFP biotinylated Ab, Novus Biologicals, Cat # NB600-310B; 100 µL/well) was added before a second 2-hour incubation. Streptavidin-HRP (1:200 in reagent diluent; R&D Systems, Cat # DY998) was added (100 µL/well) and the plate was incubated for 20 minutes at room temperature. Turbo TMB substrate (Thermo Scientific, Cat # 34022) was added (100 µL/well) and the plate was incubated for 20 minutes at room temperature. Following incubation, 100 µL/well 1N HCl was added to stop the reaction. Optical density was read

at 450 and 570 nm with a spectrophotometer. To express the amount of EGFP in terms of total protein present in the samples, a BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Prod # 23227) was run concurrent with each ELISA in accordance with the manufacturer's protocol.

### Analysis and Statistics

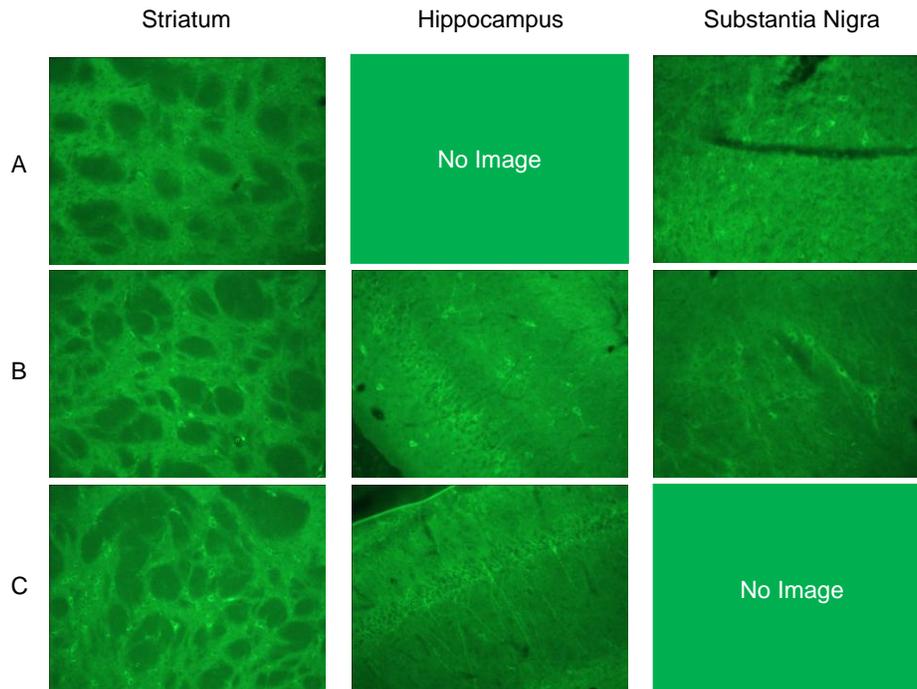
Statistical analyses were performed using Graph Pad Prism version 4. All group comparisons were performed using 1-way analysis of variance (ANOVA) and the source of any statistically significant differences was analyzed post-hoc using Tukey's Multiple Comparison Test ( $\alpha = 0.05$ ). Data was presented as mean  $\pm$  standard error of the mean (SEM).

## **Results**

### Characterization of Lipoplex and Liposome Formulations

Lipoplex particle sizes averaged  $277.95 \pm 50.95$  nm in diameter and had a zeta potential of  $-32.13 \pm 3.11$  mV (mean  $\pm$  SEM for 6 preparations). However, the final diameter of lipoplexes from the first preparation was 527.6 nm, a value greater than the mean plus three standard deviations of the remaining values. Treating this preparation as an outlier resulted in average lipoplex particle sizes of  $228.02 \pm 12.44$  nm and an average zeta potential of  $-32.73 \pm 3.74$  mV. Empty liposomes were determined to have an average size of approximately 150 nm in diameter with an approximate zeta potential of +70 mV for the first preparation. The characteristics of the empty liposomes were not measured for subsequent preparations.

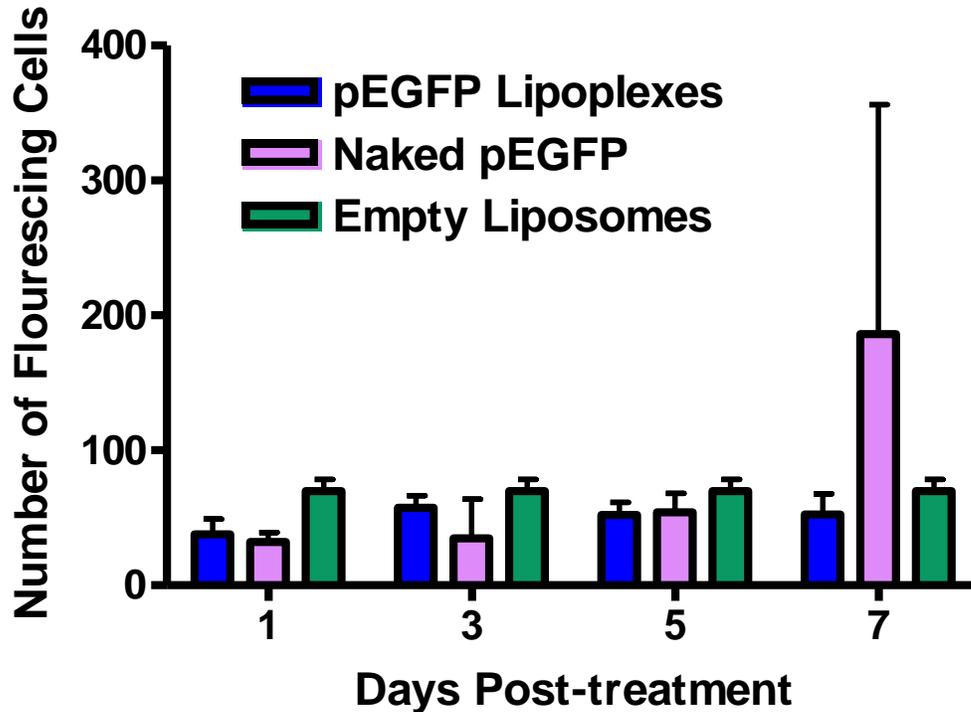
Detection of EGFP Expression by Fluorescence Microscopy



**Figure 1. Microscopic images of green fluorescent cells:** Green fluorescent cells in the striatum, hippocampus, and substantia nigra of rats that received (A) empty liposomes (B) naked pEGFP and (C) pEGFP lipoplexes. All images taken at 20X magnification.

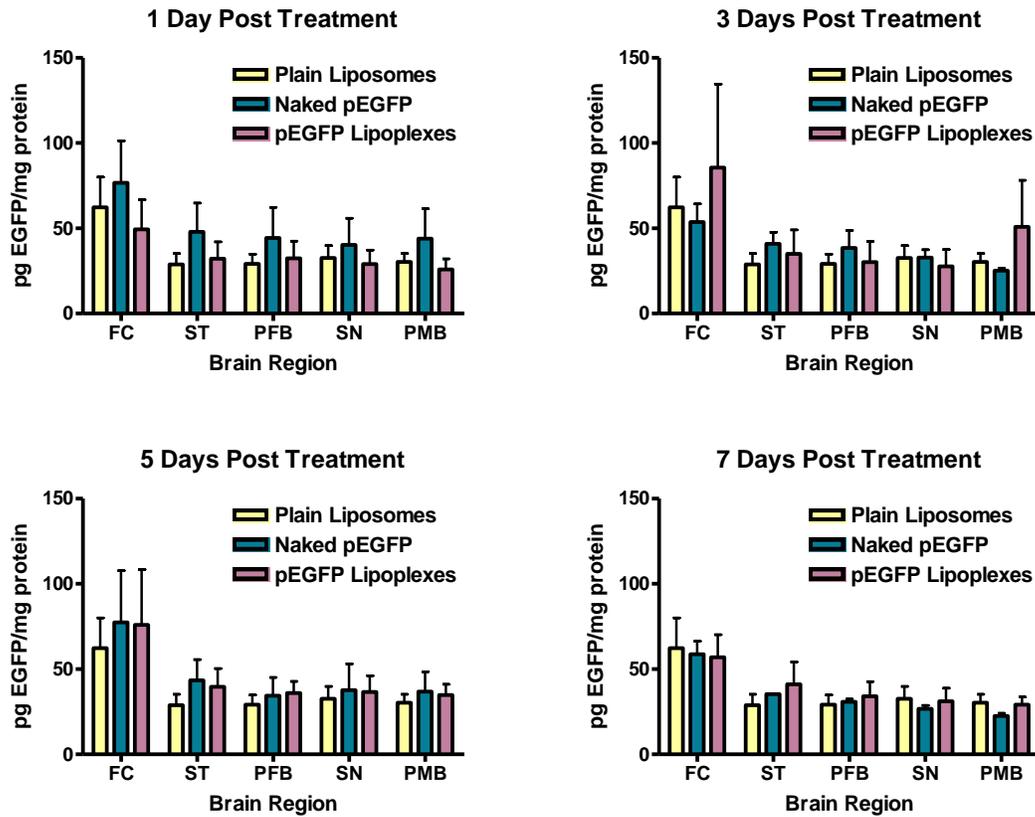
The distribution of green fluorescent cells throughout the brain was largely uniform although dense clusters of green fluorescent cells were often observed at the level of the striatum, in the hippocampus, particularly in the region of the dentate gyrus, and in the substantia nigra (Fig. 1). However, there were no significant differences in the total number of green fluorescent cells seen in the examined brains for rats which received pEGFP lipoplexes, naked pEGFP, and plain liposome controls (Fig. 2). The apparent increase in fluorescent cells at 7 days post-treatment in rats given naked pEGFP was due to a high value in one animal of the group (N = 2; the low N was due to the fact that one animal allocated to the 7 days post-treatment group was inadvertently sacrificed at 3 days post-treatment). Due to the large variability in the number of

fluorescent cells in these animals, the apparent increase was not statistically significant ( $F[11] = 0.421, P > 0.5$ ).

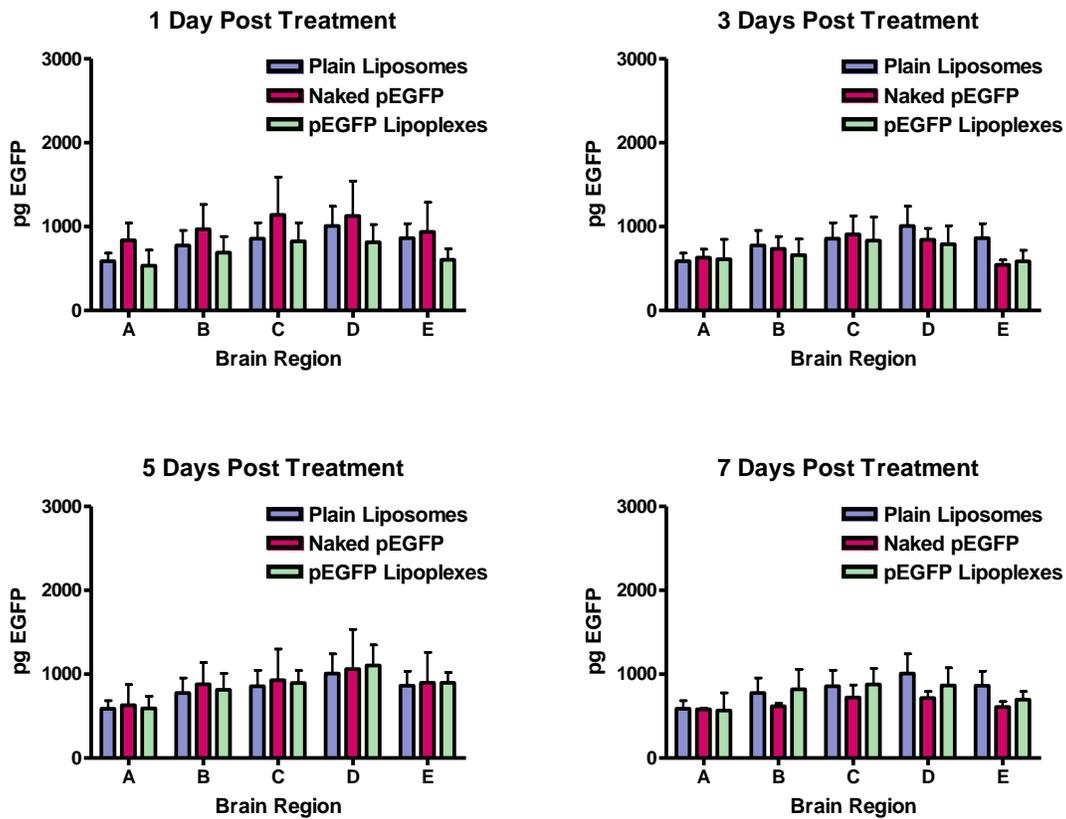


**Figure 2. Green fluorescent cells counted under microscope:** The number of green fluorescent cells as counted under microscope at 20X magnification did not vary significantly between animals that received pEGFP lipoplexes, naked pEGFP, or empty liposomes. There was also no significant variation with relation to the number of days post IN delivery. As a result of an inadvertent error that resulted in allocation of one animal to the wrong group, only two animals were included in the 7 days post-treatment, naked pEGFP group. This was the cause for both the seemingly high value in the corresponding data and the large error associated with it.

Detection of EGFP Expression by ELISA



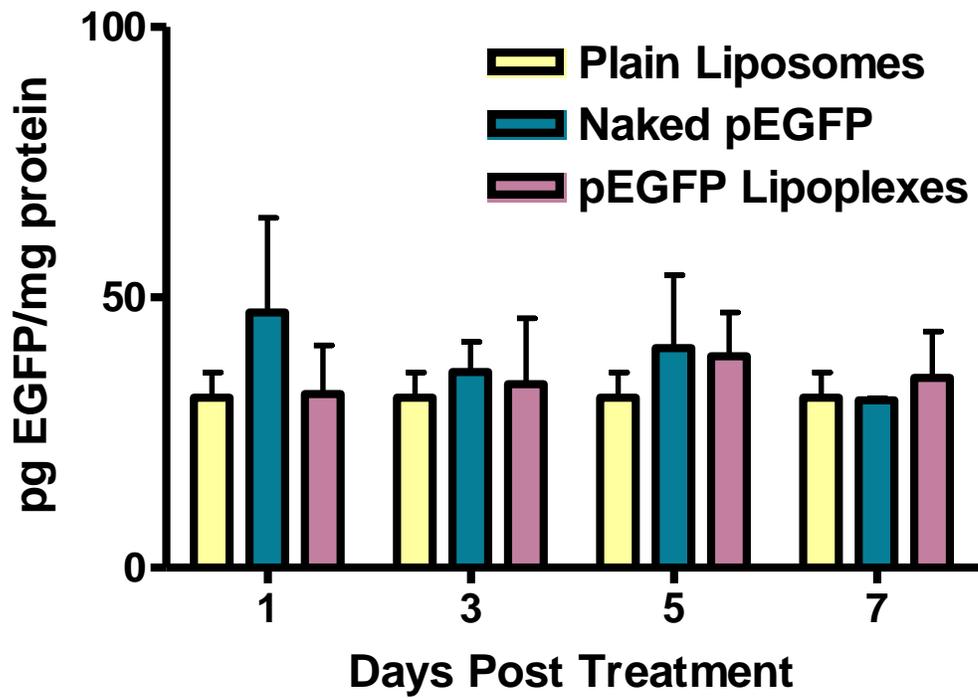
**Figure 3. EGFP per mg protein in each brain region:** ELISA was used to determine the amount of EGFP per milligram of protein in each of 5 brain regions of interest (FC, ST, PFB, SN, PMB). No significant differences in the amount of EGFP per milligram protein were found at 1, 3, 5, or 7 days post IN delivery between animals that received plain liposomes, naked pEGFP, or pEGFP lipoplexes.



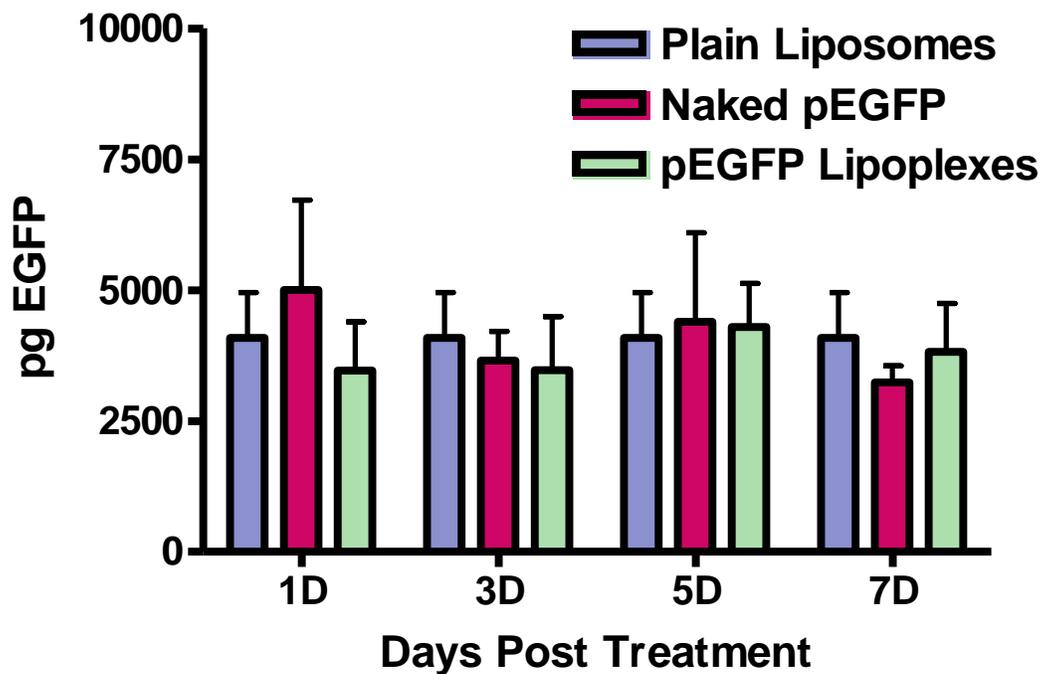
**Figure 4. EGFP in each brain region:** ELISA was used to determine the amount of EGFP in each of 5 brain regions of interest (FC, ST, PFB, SN, PMB). No significant differences in the amount of EGFP were found at 1, 3, 5, or 7 days post IN delivery between animals that received plain liposomes, naked pEGFP, or pEGFP lipoplexes.

Analysis of EGFP expression by ELISA revealed that there was no statistically significant difference in the apparent levels of EGFP between rostral to caudal brain regions (FC, ST, PFB, SN, PMB: Fig. 3 and 4). This was true for all treatment groups at 1, 3, 5, and 7 days post treatment when the data was expressed as pg EGFP per mg protein (Fig. 3), or when expressed as pg EGFP per brain region (Fig. 4). However, it should be noted that the sensitivity of the ELISA for EGFP was not as high as expected since even samples which had no EGFP present (i.e. the plain liposome group) yielded values in the assay of 25 to over 50 pg EGFP/mg protein. The lack of sensitivity made it difficult to

assess whether any EGFP was expressed in the pEGFP treatment groups (i.e. pEGFP lipoplexes and naked pEGFP).



**Figure 5. EGFP per mg protein in whole brain:** ELISA was used to determine the amount of EGFP per milligram protein in whole brains. No significant differences in the amount of EGFP per milligram protein were found between rats that received plain liposomes, naked pEGFP, or pEGFP lipoplexes nor were such differences found relative to the number of days post IN delivery.



**Figure 6. EGFP in whole brain:** ELISA was used to determine the amount of EGFP in whole brains. No significant differences in the amount of apparent EGFP were found between rats that received plain liposomes, naked pEGFP, or pEGFP lipoplexes nor were such differences found relative to the number of days post IN delivery.

Whole brain EGFP levels also showed no statistically significant difference for groups that received pEGFP lipoplexes, naked pEGFP, and plain liposome controls. Even in animals that received plain liposomes, the assay showed detection of apparent EGFP (Fig. 5 and 6).

## Discussion

### Interpretation of Results

Despite past successes with lipoplex vectors for gene therapy and the successful delivery of proteins to the brain via the intranasal route of administration, the current study did not achieve success using these methods in concert. While fluorescence

microscopy did reveal a diffuse presence of green fluorescent cells in the brains of animals that received both naked pEGFP and pEGFP lipoplexes, similar numbers and patterns of green fluorescing cells were also observed in animals which received plain liposomes. The presence of green fluorescent cells in animals which did not receive any form of EGFP plasmid DNA suggests that the fluorescence was a consequence of some other factor. One possible explanation is that green fluorescence may be a result of tissue fixation with paraformaldehyde, as was used to prepare the tissue of animals in the fluorescence microscopy portion of this study.

In fact, tissue fixation using formaldehyde, generated from paraformaldehyde powder, was used as a means of identifying catecholaminergic cells in a method developed by Falck and Hillarp in 1962 [28-31]. The green fluorescent products of the condensation reaction between catecholamines and formaldehyde represented the first time that neurotransmitters were directly visualized using a microscope [29], and the method has since been used to identify catecholamine containing neurons, including dopamine neurons [30], as well as other biogenic monoamines and their chemical precursors [29] in a variety of tissues [28-32]. Thus, it is possible that paraformaldehyde condensation of catecholamines and other biogenic monoamines was responsible for some, if not most, of the green fluorescent cells observed in this study. Studies using GFP and EGFP as fluorescent markers in tissues such as brain should therefore use a method other than fluorescence for detecting the protein.

ELISA is one method which uses antibody recognition of the protein as the means of detection. Therefore, levels of EGFP were also measured in the study using

ELISAs. However, the amount of apparent EGFP detected in tissue from animals which received only plain liposomes was again very similar to that found in the brains of animals that received intranasal naked pEGFP and pEGFP lipoplexes. As was the case for fluorescence microscopy, this suggested that some of the apparent EGFP was due to non-specific detection of some other agent in the ELISA and did not indicate the presence of EGFP. Although the capture and detection antibodies used were marketed as a matched-pair for use in sandwich ELISA by Novus Biologicals, a certain degree of cross reactivity (i.e. binding and detection of the capture antibody by the detection antibody) could not be ruled out and may have been responsible for the erroneous detection of EGFP. Another possible explanation for the apparent detection of EGFP, even in brains not exposed to the EGFP plasmid, could be the interaction of one of the detection reagents in the ELISA protocol, such as the avidin-HRP conjugate, with a substance present in brain, such as biotin. These confounding variables may have limited sensitivity of the assay and made detection of authentic EGFP difficult or impossible, especially since low levels of expression were expected.

Another potentially confounding factor was encountered after running three of the six ELISA plates. The intensity of the final reaction product generated by HRP in the last three plates was reduced by a factor of approximately ten. The change was readily observable by the naked eye but was initially regarded as irrelevant since any variability at the final step should be accounted for by the presence of standards on each plate. However, the inherent limit of detectable absorbance imposed by the spectrophotometer may have caused the standard curves from the final three plates,

and thus the data in its entirety, to be compromised. Subsequent testing revealed that the decreased intensity of the reaction product was likely a result of degradation of the Turbo TMB substrate used (Thermo Scientific, Cat # 34022). Changing the substrate to SureBlue TMB (KPL, Cat # 52-00-01) subsequently remedied the problem but not until after the above data had been collected.

### *Implications of Findings*

Besides the issues regarding the sensitivity of the assay, several other factors may have impacted the ultimate lack of gene expression in this study. These include lack of adequate transport of lipoplexes into the brain across the nasal epithelium, endocytosis, and nuclear uptake. Of these, insufficient transport of lipoplexes into the brain represents the most immediate issue since neither of the subsequent events may take place without it. However, other studies have shown that the intranasal route of administration does allow passage into the brain of large biomolecules, such as proteins, that would not normally cross the blood brain barrier [20, 22, 24-25, 33-34]. Furthermore, previous work in the sponsor's laboratory has demonstrated successful delivery of the protein Alexa 488-OVAL [22] as well as GDNF to the brain [33-34]. While both liposomal and non-liposomal preparations resulted in a diffuse distribution of Alexa 488-OVAL in the brain, the use of cationic liposomes, very similar to the lipoplexes used in this study, resulted in enhanced transport of the protein to the brain, prolonged presence in the brain, and apparent cellular uptake by dopaminergic neurons. Thus, it is likely that a portion of the pEGFP lipoplexes also entered the brain following intranasal administration, although they did not achieve detectable transfection. However, since

an assay (e.g. Southern blot) was not performed to determine the presence and location of the EGFP gene, we cannot be sure of this.

Even if lipoplexes had successfully entered the brain, endocytosis and nuclear uptake, still pose significant biological barriers that may have hindered transfection efficacy. The mechanism by which lipoplexes and other lipid vectors gain entry into cells is not yet well understood but may occur via several distinct endocytotic processes [14]. These include clathrin- and caveolin-mediated endocytosis as well as macropinocytosis and phagocytosis [14]. In each scenario, the positively-charged lipid vector is believed to aid in its association with the negatively-charged cellular membrane resulting in the ultimate formation of an endosome that contains the lipoplex [14]. Once endocytosed, the fate of the lipoplex is subject to possible degradation by the other contents of the endosome as well as potential encounters with lysosomes. Furthermore, dissociation of the plasmid DNA from the lipid complex does not occur spontaneously and lipoplexes must be engineered to promote appropriate DNA release. Methods for achieving this include formulating the lipoplex with high concentrations of compounds such as sucrose to promote osmotic swelling and lysis, creating pH sensitive lipoplexes that lyse in the lower pH of the endosome, or incorporating peptides into the lipoplexes that promote membrane lysis.

Assuming the lipoplexes are endocytosed and the DNA is released from both the endosome and the lipid complex, the nucleus remains arguably the most formidable barrier to transfection. In most cells, the cell cycle is believed to play an essential role in the uptake of plasmid DNA by the nucleus. The temporary breakdown of the nuclear

membrane that occurs during mitosis allows a direct route for transfection. However, in post-mitotic cells such as neurons, the nuclear membrane remains intact and the entry of plasmids into the nucleus cannot occur via this mechanism. Yet, the transfection of neurons [18] as well as other post-mitotic cells, such as those of the respiratory epithelium [35], has been achieved suggesting that an alternate mechanism of nuclear uptake, that isn't dependent on the cell cycle, also occurs. Such a mechanism is likely to rely on active transport, but it remains unclear as to how the relatively large plasmid DNA transverses the smaller nuclear pores.

### Conclusion

Ongoing work in the sponsor's laboratory will focus on improving several of the problems encountered in this study. Efforts are already being made to optimize the current ELISA protocol in order to achieve the highest possible sensitivity and specificity for EGFP. Additionally, future studies will evaluate the efficacy of polyethylene glycol (PEG)-ylated polylysine vectors as an alternative to lipoplex vectors for plasmid DNA delivery. Recent studies have shown that these vectors may overcome some of the issues associated with transfection of post-mitotic cells [18, 35]. The results from this study, along with the eventual results using PEGylated polylysine vectors, will help to elucidate problems with IN delivery of genes so that the current paradigm can be improved and hopefully used to deliver a GDNF-encoding plasmid to the brain. Transport of therapeutic genes across the nasal epithelium and into the brain represents only the first of several barriers along the pathway to transfection. The mechanisms mediating endocytosis and nuclear uptake of vectors/genes must be better

understood for true progress to be made. Then, we can adequately evaluate the use of IN gene delivery for the treatment of debilitating CNS disorders such as Parkinson's disease.

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