

Monoclonal Antibody-GDNF Fusion Protein Penetrates the Blood-Brain Barrier in the Mouse

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Abbreviations: BBB, blood-brain barrier; MAb, monoclonal antibody; GDNF, glial derived neurotrophic factor; PD, Parkinson's disease; TfR, transferrin receptor; cTfRMAb, chimeric MAb against the mouse TfR; HIR, human insulin receptor; HIRMAb, engineered MAb against the HIR; ID, injected dose; HC, heavy chain; LC, light chain; VH, variable region of the HC; VL, variable region of the LC; TV, tandem vector; SFM, serum free medium; MTX, methotrexate; CHO, Chinese hamster ovary; RT, room temperature; TCA, trichloroacetic acid; RRA, radio-receptor assay; NSB, non-specific binding; VD, volume of distribution; PK, pharmacokinetics; PS, permeability-surface area; AUC, area under the curve; AA, amino acid; TH, tyrosine hydroxylase

Abstract

Glial-derived neurotrophic factor (GDNF) is a potent neuroprotective agent for multiple brain disorders, including Parkinson's disease. However, GDNF drug development is difficult, because GDNF does not cross the blood-brain barrier (BBB). To enable future drug development of GDNF in mouse models, the neurotrophin was re-engineered as an IgG fusion protein to enable penetration through the BBB following intravenous administration. The 134 amino acid GDNF was fused to the heavy chain of a chimeric monoclonal antibody (MAb) against the mouse transferrin receptor (TfR), designated the cTfRMAb. This antibody undergoes receptor-mediated transport across the BBB and acts as a molecular Trojan horse to ferry the GDNF into mouse brain. The cTfRMAb-GDNF fusion protein was expressed by stably transfected Chinese hamster ovary cells, affinity purified, and the biochemical identity was confirmed by mouse IgG and GDNF Western blotting. The cTfRMAb-GDNF fusion protein was bi-functional and bound with high affinity to both the GDNF receptor (GFR)- $\alpha 1$, $ED_{50}=1.7 \pm 0.2$ nM, and the mouse TfR, $ED_{50}=3.2 \pm 0.3$ nM. The cTfRMAb-GDNF fusion protein was rapidly taken up by brain, and the brain uptake was 3.1 ± 0.2 % injected dose/gram brain at 60 minutes after intravenous injection of a 1 mg/kg dose of the fusion protein. Brain capillary depletion analysis demonstrated the majority of the fusion protein was transcytosed across the BBB with penetration into brain parenchyma. The brain uptake results indicate it is possible to achieve therapeutic elevations of GDNF in mouse brain with intravenous administration of the cTfRMAb-GDNF fusion protein.

Introduction

Glial derived neurotrophic factor (GDNF) is a potential treatment for multiple brain disorders including Parkinson's disease (PD), stroke, and addiction (Lapchak et al, 1997; Ron and Janak, 2005; Boado et al, 2008). However, GDNF does not cross the blood-brain barrier (BBB) in the mouse (Kastin et al, 2003) or the Rhesus monkey (Boado and Pardridge, 2009a). Consequently, the neurotrophin was administered by direct cranial infusion in patients with PD (Lang et al, 2006). However, the clinical trial was not successful, and subsequent studies demonstrated limited penetration of GDNF into brain parenchyma following trans-cranial infusion (Salvatore et al, 2006). An alternative approach to GDNF drug development is the re-engineering of the neurotrophin as a fusion protein with a BBB molecular Trojan horse (Pardridge, 2008). The latter is a peptidomimetic monoclonal antibody (MAb) against an endogenous BBB peptide receptor transport system, such as the BBB insulin receptor or transferrin receptor (TfR). The MAb undergoes receptor-mediated transport across the BBB, without interference of endogenous peptide transport. The MAb acts as a molecular Trojan horse to ferry a fused neurotherapeutic across the BBB following systemic administration of the fusion protein.

A fusion protein of GDNF and a genetically engineered MAb against the human insulin receptor (HIR) has been engineered (Boado et al, 2008), and the HIRMAb-GDNF fusion protein penetrates the primate BBB in vivo, whereas native GDNF does not cross the primate BBB (Boado and Pardridge, 2009a). The HIRMAb-GDNF fusion protein retains high affinity binding to both the HIR and the GDNF receptor (GFR)- α 1, and is equi-potent with recombinant GDNF in GFR α 1 receptor binding or bio-assays in

human neural cells (Boado et al, 2008). However, the HIRMAb-GDNF fusion protein cannot be tested in rodents, because the HIRMAb part of the fusion protein is only active in humans and Old World primates, such as the Rhesus monkey (Pardridge et al, 1995). There is no known MAb against the mouse or rat insulin receptor ecto-domain that could be used as a BBB molecular Trojan horse. Therefore, a surrogate Trojan horse is used in rodents, which is a MAb against the TfR. The murine OX26 MAb against the rat TfR is used in rats (Pardridge et al, 1991); this MAb is not active against the mouse TfR (Lee et al, 2000). The rat 8D3 MAb against the mouse TfR is used for BBB drug delivery in the mouse (Lee et al, 2000). A chimeric form of the 8D3 TfRMAb has been engineered, in which the variable region of the heavy chain (VH) and the variable region of the light chain (VL) of the rat 8D3 TfRMAb were fused to the constant regions of the mouse IgG1 heavy chain and mouse kappa light chain, respectively (Boado et al, 2009). The chimeric TfRMAb, designated cTfRMAb, is 85% mouse amino acid sequence, which allows for chronic administration in mouse models.

The purpose of the present study was to engineer, express, and validate a new IgG-GDNF fusion protein that would be active in mouse models of brain disease. The new fusion protein is comprised of the cTfRMAb Trojan horse and GDNF, and this fusion protein is designated cTfRMAb-GDNF. Human GDNF was used since there is a 93% amino acid identity between human and mouse GDNF. The human GDNF monomer is fused to the carboxyl terminus of the heavy chain of the cTfRMAb, which places the neurotrophin in its native dimeric configuration (Figure 1). Following expression in stably transfected Chinese hamster ovary (CHO) cells, the cTfRMAb-GDNF fusion protein was examined with respect to binding to the mouse TfR and to the

GFR α 1. The plasma pharmacokinetics, metabolic stability, and brain uptake of the cTfRMAb-GDNF fusion protein in the C57BL/6 mouse was examined. The C57BL/6 mouse was investigated, since this mouse strain is generally used in murine models of experimental PD (Alvarez-Fischer et al, 2008).

Methods

Production of CHO line. A tandem vector (TV) containing within a single plasmid DNA the expression cassettes encoding the cTfRMAb heavy chain-GDNF fusion protein, the cTfRMAb light chain (LC), and the murine dihydrofolate reductase (DHFR), was engineered similar to a TV described previously (Boado et al, 2010). The cDNA encoding the 134 amino acid (AA) human mature GDNF was amplified by PCR as described previously (Boado et al, 2008), and subcloned at the 3'-end of the cTfRMAb HC to form the pcTfRMAb-GDNF tandem vector shown in Figure 2. The sequence of the TV was confirmed by bi-directional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX). The TV was linearized and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate (MTX) up to 80 nM in serum free medium (SFM). The CHO line was dilutionally cloned at 1 cell/well, and high producing clones were selected by measurement of medium mouse IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through multiple generations, and produced medium IgG levels of 5-10 mg/L in shake flasks at a cell density of 1-2 million cells/mL.

Protein purification. The CHO cells were propagated in 1 L bottles, until 2.4L of conditioned serum free medium was collected. The medium was ultra-filtered with a 0.2 μ m Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany), and applied to a 25 mL protein G Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH=7.1. The column was

washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH=7.1, and the fusion protein was eluted with 0.1 M glycine/pH=2.8. The acid eluate was pooled and neutralized to pH=5.5 with 1M Tris base, and concentrated with an Ultra-15 microconcentrator (Millipore, Bedford, MA) and stored sterile-filtered at 4C.

SDS-PAGE and Western blotting. The homogeneity of the cTfRMAb-GDNF fusion protein was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions using 12% and 7.5% Tris-HCl gels (Biorad Life Science, Hercules, CA), respectively. Western blot analysis was performed with a goat anti-mouse IgG (H+L) antibody (Bethyl Labs, Montgomery, TX) for the mouse IgG Western, and with a rabbit anti-human GDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for the GDNF Western. The immunoreactivity of the cTfRMAb-GDNF fusion protein was compared with the cTfRMAb described previously (Boado et al, 2009), and recombinant human GDNF (Peprotech, Rocky Hill, NJ).

GFR α 1 binding ELISA. The binding of the cTfRMAb-GDNF fusion protein to the GDNF receptor (GFR)- α 1 was determined by ELISA, as described previously (Boado et al, 2008). A mouse anti-human (MAH) IgG (Zymed-Invitrogen, Carlsbad, CA) was plated in 96-well plates overnight at 2 ug/well. Following aspiration, washing, and blocking with 1% bovine serum albumin (BSA), the Fc fusion of the human GFR α 1 extracellular domain (ECD) (R&D Systems, Minneapolis, MN) was plated at 0.4 ug/well. Following incubation at room temperature (RT) for 60 min, the wells were washed, and either the cTfRMAb-GDNF fusion protein, or mouse IgG1 κ (Sigma Chemical Co., St. Louis, MO), was plated for 2 hours at RT. Following washing, a goat anti-GDNF

antibody (R&D Systems) was plated at 0.4 ug/well for 30 min at RT. Following washing, a conjugate of alkaline phosphatase (AP) and a rabbit anti-goat (RAG) IgG(H+L) (Vector Labs) was plated and detection at 405 nm was performed with an ELISA plate reader after color development with para-nitrophenylphosphate (Sigma Chemical Co.). The ED50 of fusion protein binding to the GFR α 1 was determined by non-linear regression analysis using the BMDP2007e software (Statistical Solutions, Dublin, Ireland).

SK-N-MC bio-assay. A bio-assay of GDNF activity was performed as described previously (Boado et al, 2008), using human neural SK-N-MC cells, which were dual transfected with the c-ret kinase and a luciferase reporter plasmid under the influence of the 5'-flanking sequence of the rat tyrosine hydroxylase (TH) gene (Tanaka et al, 2003). Addition of GDNF to the medium activates the TH promoter via signal transduction mediated by the GFR α 1/c-ret kinase system, and this leads to increased luciferase expression by the cells. The cells were grown in collagen coated 24-well dishes to 70% confluency and 0.16-16 nM concentrations of the cTfRMAB-GDNF fusion protein were added to the medium. After a 24 hour incubation at 37°C, the cells were extracted with 200 uL/well of Luciferase Reporter Lysis buffer (Promega, Madison, WI). Following centrifugation, luciferase enzyme activity was measured in the lysate with a luminometer, and luciferase enzyme activity, reported as nanogram (ng) of luciferase, was normalized per mg sample protein using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL). The luciferase activity (Act), ng luciferase per mg protein, was plotted vs the concentration (S) of the cTfRMAB-GDNF fusion protein in the medium, and the data was fit to the following equation, $Act = [(A_{max})(S)/(EC50+S)]$, where A_{max} is the maximal luciferase activity and ED50 is the concentration of fusion

protein that produces a 50% increase in luciferase activity. The data were fit by non-linear regression using the BMDP2007e statistical software.

Mouse transferrin receptor radio-receptor assay. The binding of the cTfRMAb-GDNF fusion protein to the mouse transferrin receptor (TfR) was determined with a radio-receptor assay described previously (Boado et al, 2009), using [125 I]-labeled rat 8D3 MAb to the mouse TfR as the binding ligand. The binding of the [125 I]-8D3 MAb to the mouse TfR, expressed on the plasma membrane of mouse fibroblasts, was displaced by increasing concentrations of either unlabeled 8D3 MAb or the cTfRMAb-GDNF fusion protein. For the 8D3 self-inhibition assay, the K_D (nM), the maximal binding, B_{max} (pmol/mg protein), and the non-saturable binding, NSB (fmol/mg protein) were computed. For the cTfRMAb-GDNF cross-competition assay, the K_I (nM) and the NSB were computed. Parameters estimates were made by non-linear regression analysis as described previously (Boado et al, 2009).

Radio-labeling of fusion protein. The cTfRMAb-GDNF fusion protein, which was injected into mice for a pharmacokinetics analysis, was radiolabeled with [3 H]-N-succinimidyl propionate (NSP) from American Radiolabeled Chemicals (St. Louis, MO), as described previously (Boado and Pardridge, 2009a). The labeled protein was purified with a 1x28 cm Sephadex G-25 gel filtration column, with an elution buffer of 0.01 M sodium acetate/0.15 M NaCl/pH=5.5. The cTfRMAb-GDNF fusion protein was labeled to a specific activity of 0.40 μ Ci/ μ g and a trichloroacetic acid (TCA) precipitability of >99%.

Pharmacokinetics and brain uptake in the mouse. Adult male C57BL/6J mice, weighing 26 grams, were obtained from Jackson Labs (Bar Harbor, ME). All

procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Mice in groups of 3 were anesthetized with intra-peritoneal (IP) ketamine (100 mg/kg) and xylazine (10 mg/kg), and injected either intravenously (IV) in the tail vein or IP with 0.1 mL (10 uCi) of [³H]-cTfRMAB-GDNF fusion protein. The injection dose in each mouse of the cTfRMAB-GDNF fusion protein was 1.0 mg/kg. An aliquot (50 uL) of blood was collected at 0.25, 2, 5, 15, 30, and 60 min from 3 mice at each time point after injection of the fusion protein. The venous blood was sampled, in 50 uL aliquots, from a PE10 cannula inserted in the external jugular vein, which was maintained patent with heparinized saline. The blood was centrifuged for collection of plasma, which was analyzed for radioactivity. At 60 min after injection, the mice were euthanized and the cerebral hemispheres were removed and weighed for each mouse. One hemisphere was used for total radioactivity after solubilization in Soluene-350 (Perkin Elmer, Downers Grove, IL), and one hemisphere was homogenized for capillary depletion analysis. Plasma and tissue samples were analyzed for ³H radioactivity with Optifluor-O (Perkin Elmer) and a liquid scintillation counter (Tricarb 2100TR, Perkin Elmer). Brain uptake data was expressed as a volume of distribution (VD), which is the ratio of the 60 min organ radioactivity (DPM/gram) divided by the 60 min plasma radioactivity (DPM/uL), or as % of injected dose (ID)/gram tissue. The plasma radioactivity that was precipitable with cold 10% TCA was determined at each time point.

The plasma radioactivity, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a bi-exponential equation,

$$\%ID/mL = A1e^{-k1t} + A2e^{-k2t}$$

The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the pharmacokinetics (PK) parameters, including the median residence time (MRT), the central volume of distribution (Vc), the steady state volume of distribution (Vss), the area under the plasma concentration curve (AUC), and the systemic clearance (CL). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software. Data were weighted by $1/(\%ID/mL)^2$.

The organ clearance ($\mu L/min/g$), also called the BBB permeability-surface area (PS) product, is computed from the terminal brain uptake ($\%ID/g$) and the 60 min plasma AUC ($\%IDmin/mL$) as follows:

$$PS \text{ product} = [(\%ID/g)/AUC] \times 1000$$

Capillary depletion method. One cerebral hemisphere from each mouse was processed for the capillary depletion method as described previously (Triguero et al, 1990). Mouse brain, ~0.2 gram, was homogenized in a 4-fold volume of cold physiologic buffer with a glass tissue grinder. The solution was made 20% dextran (60-80 kDa, Sigma) and centrifuged at 3200g at 4C for 10 min. The fatty layer was transferred to a new tube, followed by removal of the post-vascular supernatant, which was pooled with the fatty layer. The capillary pellet was resuspended in 1.0 mL water. Aliquots of the initial dextran homogenate, the pooled post-vascular supernatant, and the vascular pellet were analyzed for 3H radioactivity using Soluene-350 and Optifluor-O as described above. The volume of distribution (VD) was determined for each of the 3 fractions from the ratio of total 3H radioactivity in the fraction, DPM/gram brain, divided by the 3H radioactivity in the 60 min terminal plasma, DPM/ μL . A high VD in the post-vascular supernatant, as compared to the VD in the capillary pellet, is evidence for

transport of the protein through the BBB into brain parenchyma. The use of the capillary depletion method to assay BBB transcytosis in vivo is valid only when the study protein is bound with high affinity to the BBB receptor, as represented by a low nM binding constant (Triguero et al, 1990).

Results

A tandem vector was engineered, which contained the expression cassettes for the heavy chain fusion gene, the light gene, and the DHFR gene on a single strand of DNA. DNA sequence analysis showed the 3 expression cassettes spanned 6,490 nucleotides (nt). The light chain was comprised of 234 AA, which included a 20 AA signal peptide, a 108 AA variable region of the light chain (VL) of the cTfRMAb, and a 106 AA mouse kappa light chain constant region, which is 100% identical to AA 113-218 of BAA06141. The predicted molecular weight of the light chain is 23,554 Da with a predicted isoelectric point (pI) of 5.73. The fusion protein of the cTfRMAb heavy chain and the GDNF was comprised of 597 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 64,018 Da with a predicted pI of 8.18. The domains of the fusion heavy chain include a 118 AA variable region of the heavy chain (VH) of the cTfRMAb, a 324 AA mouse IgG1 constant region, which is 100% identical to AA 140-463 of BAC44885, a 2 AA linker (Ser-Ser), and the 134 AA human GDNF, which is 100% identical to AA 78-211 of NP_000505. The HC contains 3 N-linked glycosylation sites: 1 site in the constant region of the IgG heavy chain and 2 sites in the GDNF domain.

The CHO-derived cTfRMAb-GDNF fusion protein migrated as a doublet on reducing SDS-PAGE with a ~65 kDa heavy chain (HC) and a ~30 kDa light chain (LC) (Figure 3A), and migrated as a 190 kDa protein on non-reducing SDS-PAGE (Figure 3B). Western blot analysis with a primary antibody against mouse IgG detected both the HC and the LC of the cTfRMAb and the cTfRMAb-GDNF fusion protein, but did not react with human GDNF (Figure 4A). Western blot analysis showed a primary antibody

against human GDNF reacted with both the HC of the cTfRMAb-GDNF fusion protein and with GDNF, but did not react with HC of the cTfRMAb (Figure 4B).

The 8D3 MAb was radio-iodinated and used as the ligand for the mouse TfR RRA with mouse fibroblasts, and a self-inhibition curve was saturable and characterized by a KD of 3.8 ± 0.7 nM and a Bmax of 0.23 ± 0.04 pmol/mg protein (Figure 5A). The KI of inhibition of binding of the ^{125}I -8D3 MAb by unlabeled cTfRMAb-GDNF fusion protein was 3.2 ± 0.3 nM (Figure 5B), which was comparable to the KD of 8D3 binding to the mouse TfR. The cTfRMAb-GDNF fusion protein retained high affinity binding to the recombinant human GFR α 1 with an ED50 of 1.7 ± 0.2 nM; conversely, there was no binding of mouse IgG1 to the GFR α 1 (Figure 6). The cTfRMAb-GDNF fusion protein exhibited GDNF biological activity and stimulated luciferase gene expression in SK-N-MC neural cells co-transfected with the c-ret kinase and the luciferase gene under the influence of the TH promoter (Figure 7). The ED50 in the bio-assay, 1.4 ± 0.7 nM (Figure 7), approximated the ED50 of binding in the GFR α 1 ELISA, 1.7 ± 0.2 nM (Figure 6).

The cTfRMAb-GDNF fusion protein was radiolabeled with the ^3H -NSP reagent (Methods), and injected into adult male C57Bl/6J mice via either IV or IP administration. The clearance of the [^3H]-cTfRMAb-GDNF fusion protein from plasma after IV administration, and the appearance of plasma radioactivity after IP administration of the fusion protein, is plotted in Figure 8. The fusion protein entered the blood compartment poorly following IP administration (Figure 8). For the IV injection studies, the plasma radioactivity decay curve was fit to a bi-exponential equation to yield the pharmacokinetics (PK) parameters shown in Table 1. The [^3H]-cTfRMAb-GDNF fusion

protein was metabolically stable after IV administration, as the plasma radioactivity that was precipitable by TCA was 98.2 ± 0.1 , 97.1 ± 0.1 , 96.7 ± 0.8 , 96.3 ± 0.7 , 95.4 ± 0.1 , and 92.5 ± 0.8 , respectively at 0.25, 2, 5, 15, 30, and 60 min after IV injection.

The uptake of the [^3H]-cTfRMAB-GDNF fusion protein by brain was determined at 60 min after IV injection, and was 3.1 ± 0.2 %ID/gram brain, which is high compared to the brain uptake of a brain blood volume marker in the mouse, such as the OX26 MAb to the rat TfR (Figure 9A). The BBB permeability-surface area (PS) product of the cTfRMAB-GDNF fusion protein, which is equal to the ratio of the 60 min %ID/g (Figure 9A) and the 60 min plasma AUC (Table 1), is 3.0 ± 0.2 uL/min/g. The capillary depletion analysis showed the VD of the cTfRMAB-GDNF fusion protein in the brain homogenate was high, 244 ± 19 uL/gram (Figure 9B), which is 23-fold higher than the brain VD for the OX26 MAb in the mouse, 10.7 ± 0.6 uL/gram (Lee et al, 2000). The VD of the fusion protein in the post-vascular supernatant, 147 ± 33 uL/gram (Figure 9B), is 60% of the homogenate VD, indicating the majority of the cTfRMAB-GDNF fusion protein had completed transcytosis through the BBB by 60 min after IV injection. The radioactivity in the post-vascular brain supernatant that was precipitable by TCA was 98.1 ± 0.1 % at 60 min after IV injection, demonstrating the product appearing in the brain parenchyma was intact cTfRMAB-GDNF fusion protein.

Discussion

The results of these studies are consistent with the following conclusions. First, the genetic engineering of the tandem vector (Figure 2) allowed for the isolation of a high producing, stably transfected CHO line that secretes the hetero-tetrameric cTfRMAb-GDNF fusion protein in serum free medium. Second, the secreted cTfRMAb-GDNF fusion protein is correctly processed by the CHO cells based on SDS-PAGE and mouse IgG and human GDNF Western blotting (Figures 3-4). Third, the cTfRMAb-GDNF fusion protein is a bi-functional protein with dual receptor specificity, as demonstrated by binding assays for both the mouse TfR and the GFR α 1 (Figures 5-6), and neural cell bio-assay (Figure 7). Fourth, the brain uptake of the cTfRMAb-GDNF fusion protein is high, 3.1 ± 0.2 % ID/gram brain (Figure 9A), and the capillary depletion analysis indicates the majority of the cTfRMAb-GDNF fusion protein taken up by brain has penetrated the BBB and entered brain parenchyma (Figure 9B).

There are multiple potential approaches to the genetic engineering of an IgG-GDNF fusion protein. GDNF, similar to many other neurotrophins, is produced within the cell as a prepro-protein, and the amino terminal half of the prepro-protein is cleaved to produce the 134 AA mature GDNF. Fusion of the prepro-peptide to the amino terminus of the HC or LC of the cTfRMAb would enable the initial folding of the GDNF precursor within the host cell. However, the TfR binding sites on the antibody chains are near the amino terminus. Previous work has shown that fusion of therapeutic peptides to the amino terminus of the MAbs results in a loss of affinity of the MAb for the target BBB receptor (Boado and Pardridge, 2009b). In the present study, the mature 134 AA GDNF was fused to the carboxyl terminus of the HC of the cTfRMAb (Figure 1), which is

confirmed by the mouse IgG and GDNF Western blotting (Figure 4). Fusion of the mature GDNF to the carboxyl terminus of the HC of the cTfRMAb places the GDNF in a dimeric configuration (Figure 1), which mimics the GDNF dimer that binds the GFR α 1 (Parkash et al, 2008). The re-engineering of GDNF as the IgG fusion protein shown in Figure 1 allows for preservation of high affinity binding of GDNF to its cognate receptor. The ED50 of either cTfRMAb-GDNF fusion protein binding to the GFR α 1, 1.7 ± 0.2 nM (Figure 6), or fusion protein activation of the GFR α 1/c-ret kinase system in neural cells, 1.4 ± 0.7 nM (Figure 7), is in the low nM range. These ED50 values for the cTfRMAb-GDNF fusion protein approximate the ED50 of GDNF binding to the GFR α 1, or GDNF activation of the GFR α 1/c-ret kinase in neural cells, reported previously (Boado et al, 2008).

The cTfRMAb part of the cTfRMAb-GDNF fusion protein also retains high affinity binding to the mouse TfR with a KI of 3.2 ± 0.3 nM (Figure 5B), and this high affinity enables rapid penetration into brain via transport on the BBB TfR in the mouse in vivo. The plasma pharmacokinetics and brain uptake of the cTfRMAb-GDNF fusion protein was examined in mice in this study. The cTfRMAb-GDNF fusion protein was tritiated and administered by either IP or IV injection. The fusion protein distributes to the blood compartment poorly following IP administration, but is rapidly removed from plasma following IV injection (Figure 7). The circulating cTfRMAb-GDNF fusion protein remains intact during the first hour after IV injection, as the plasma radioactivity was 95% and 93% precipitable by TCA at 30 and 60 minutes (Results). The systemic clearance of the cTfRMAb-GDNF fusion protein, 2.11 ± 0.11 ml/min/kg (Table 1), is higher than the systemic clearance of either the rat 8D3 TfRMAb (Lee et al, 2000), or the genetically

engineered cTfRMAb (Boado et al, 2009) in the mouse. The higher systemic clearance of the cTfRMAb-GDNF fusion protein may be related to the cationic charge of the GDNF, as this neurotrophin is rapidly removed from blood following IV injection (Boado and Pardridge, 2009a).

The brain uptake of the cTfRMAb-GDNF fusion protein is high, 3.1 ± 0.2 % ID/gram brain (Figure 9A), and is comparable to the high brain uptake of a fusion protein of the cTfRMAb and a single chain Fv antibody, which is 3.5 ± 0.3 % ID/gram brain (Boado et al, 2010). In contrast, the brain uptake in the mouse of a conjugate of paclitaxel and angiopep-2, a putative ligand for the BBB low density lipoprotein receptor related protein (LRP)-1, is 10-fold lower, 0.34 % ID/gram brain (Thomas et al, 2009). The high mouse brain uptake of the cTfRMAb fusion proteins is attributed to rapid BBB transport in vivo. The rapid BBB transport correlates with the low nM constant of cTfRMAb-GDNF fusion protein binding to the mouse TfR (Figure 5). Binding of the cTfRMAb-GDNF fusion protein to the BBB TfR triggers transcytosis through the BBB and penetration of brain parenchyma. This is demonstrated by the capillary depletion method, which shows the VD of the fusion protein in the post-vascular supernatant is 60% of the total homogenate VD (Figure 9B). This finding indicates the majority of the cTfRMAb-GDNF fusion protein that has bound the BBB TfR has crossed the BBB and entered brain.

The injection dose (ID) of the cTfRMAb-GDNF fusion protein in this study was 1.0 mg/kg (Methods). Since the brain uptake of the fusion protein at this dose is 3.1 % ID/g, the brain concentration of the cTfRMAb-GDNF fusion protein is 750 ng/gram brain. The GDNF moiety constitutes 17% of the fusion protein, based on amino acid

composition (Results). Therefore, the brain concentration of exogenous GDNF is 125 ng/gram following an IV injection of 1.0 mg/kg of the cTfRMAb-GDNF fusion protein. This concentration of cerebral GDNF represents a substantial increase in brain GDNF as compared to the endogenous concentration. Endogenous GDNF has been measured in brain by ELISA following preparation of a brain homogenate. In the human brain, the GDNF concentration is 0.39 ng/gram and 0.68 ng/gram in the cortex and striatum, respectively (Michel et al, 2008). In the mouse, there are more disparate measurements of endogenous GDNF in the striatum, which vary from 0.3 ng/gram (Griffen et al, 2008) to 8 ng/gram (Airavaara et al, 2004). In either case, it is possible to achieve a substantial increase in brain GDNF following the systemic administration of the cTfRMAb-GDNF fusion protein. Therapeutic effects of GDNF in experimental PD are achieved with only a modest, e.g. 4-fold, increase in exogenous GDNF in the brain (Eslamboli et al, 2005). Such elevations in brain GDNF could be achieved with relatively small doses of the cTfRMAb-GDNF fusion protein considerably reduced from the dose, 1.0 mg/kg, used in the present study.

In conclusion, the present work describes the genetic engineering, expression, and validation of a novel IgG-GDNF fusion protein, which binds with high affinity both to the GFR α 1, to induce neuroprotection in the brain, and to the mouse TfR, to induce transport across the mouse BBB. The transport of the cTfRMAb-GDNF fusion protein across the mouse BBB is rapid, which generates high concentrations of biologically active, exogenous GDNF in the brain. Future work on the therapeutic and toxicologic effects of the cTfRMAb-GDNF fusion protein in the mouse, including mouse models of

PD, will complement parallel drug development of the human homologue, the HIRMAb-GDNF fusion protein (Boado et al, 2008).

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Footnotes

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Legends to Figures

Figure 1. The cTfRMAb-GDNF fusion protein is comprised of 2 heavy chains and 2 light chains. The heavy chain is formed by fusion of the variable region of the heavy chain (VH) of the rat 8D3 MAb against the mouse transferrin receptor (mTfR) to the amino terminus of mouse IgG1 constant (C)-region, and fusion of human GDNF to the carboxyl terminus of the heavy chain C-region. The light chain is formed by fusion of the variable region of the light chain (VL) of the rat 8D3 MAb to the mouse kappa light chain C-region (CL). The heavy chain C-region is comprised of 4 domains: CH1, hinge, CH2, and CH3.

Figure 2. The tandem vector expressing the cTfRMAb-GDNF fusion protein is comprised of a single strand of DNA, which includes separate expression cassettes for the LC of the cTfRMAb, the HC gene formed by fusion of the cDNA encoding the GDNF to the 3' end of the cDNA encoding the HC of the cTfRMAb, and murine dihydrofolate reductase (DHFR), to allow for amplification of cell lines with methotrexate treatment. The HC and LC genes are 5'-flanked by the cytomegalovirus (CMV) promoter, and 3'-flanked by the bovine growth hormone (BGH) polyA sequence, and the DHFR gene is 5'-flanked by the SV40 promoter, and 3'-flanked by the hepatitis B virus (HBV) polyA sequence. The plasmid also contains genes for neomycin (neo) resistance and ampicillin resistance (ampR).

Figure 3. Reducing (A) and non-reducing (B) SDS-PAGE of molecular weight standards (lane 1) and the cTfRMAB-GDNF fusion protein (lane 2). The heavy chain doublet reflects differential glycosylation, which is also seen on Western blotting (Figure 4).

Figure 4. Western blotting with a primary antibody against mouse IgG (A) or against GDNF (B). The anti-mouse antibody reacts with the HC and LC of both the cTfRMAB and the cTfRMAB-GDNF fusion protein, but does not react with GDNF. The anti-GDNF antibody reacts only with the HC of the cTfRMAB-GDNF fusion protein and with GDNF, but does not react with the HC or LC of the cTfRMAB.

Figure 5. Radio-receptor assay of the mouse TfR uses mouse fibroblasts as the source of the mouse TfR and [¹²⁵I]-8D3 as the binding ligand. Binding is displaced by unlabeled 8D3 MAb (A) or the cTfRMAB-GDNF fusion protein (B). The KD of 8D3 self-inhibition and the KI of cTfRMAB-GDNF cross-inhibition were computed by non-linear regression analysis.

Figure 6. Binding of the cTfRMAB-GDNF fusion protein to the GFR α 1 extracellular domain is saturable. The ED50 of cTfRMAB-GDNF fusion protein binding was determined by non-linear regression analysis. There is no binding to the GFR α 1 by mouse IgG1 (mIgG1).

Figure 7. The cTfRMAB-GDNF fusion protein activates luciferase gene expression in cultured SK-N-MC neural cells which are co-transfected with the c-ret kinase and the

luciferase gene under the influence of the TH promoter. Data are mean \pm S.E. (n=4 wells/point). The Amax and ED50 were determined by non-linear regression analysis.

Figure 8. Plasma concentration, expressed as % of injected dose (I.D.)/mL, of the [3 H]-cTfRMAB-GDNF fusion protein after intravenous or intraperitoneal injection in the mouse. Data are mean \pm S.E. (n=3 mice/point).

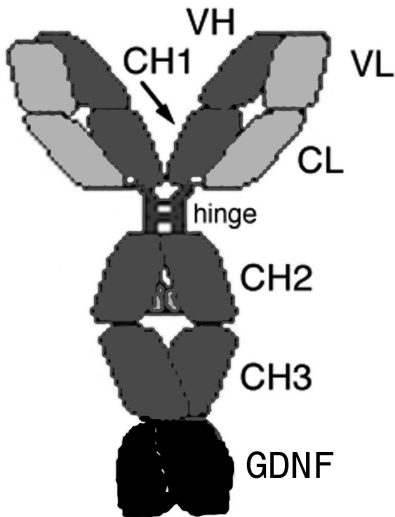
Figure 9. (A) Brain uptake, expressed as % of injected dose (ID)/gram brain, for the cTfRMAB-GDNF fusion protein, in comparison with the mouse brain uptake for the OX26 MAb against the mouse TfR, which is a brain blood volume marker in the mouse (Lee et al, 2000). (B) Volume of distribution (VD) of the cTfRMAB-GDNF fusion protein in the brain homogenate (H), the post-vascular supernatant (S), and the vascular pellet (P). Data are mean \pm S.E. (n=3 mice).

Table 1. Pharmacokinetic parameters of cTfRMAb-GDNF in the mouse

Parameter	Units	cTfRMAb-GDNF
A1	%ID/mL	22.7 ± 2.8
A2	%ID/mL	24.7 ± 0.9
k1	min ⁻¹	1.08 ± 0.29
k2	min ⁻¹	0.014 ± 0.001
MRT	min	72 ± 5
Vc	mL/kg	81 ± 4
Vss	mL/kg	154 ± 4
AUC(60 min)	%ID·min/mL	1031 ± 19
AUCss	%ID·min/mL	1822 ± 90
Cl	mL/min/kg	2.11 ± 0.11

MRT=mean residence time; Vc=plasma volume; Vss=steady state volume of distribution; AUC(60 min)=area under the curve first 60 min; AUCss=steady state AUC; Cl=clearance from plasma. The injection dose was 1.0 mg/kg, and the body weight of the mice was 0.026 kg.

Tf receptor



GFR α 1

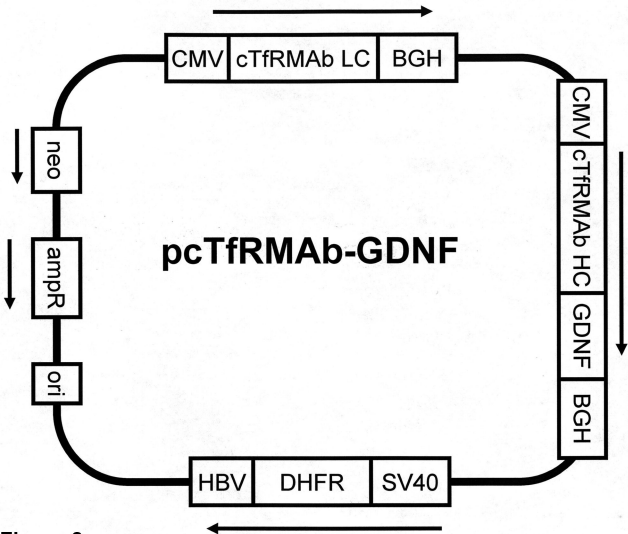


Figure 2

Figure 3

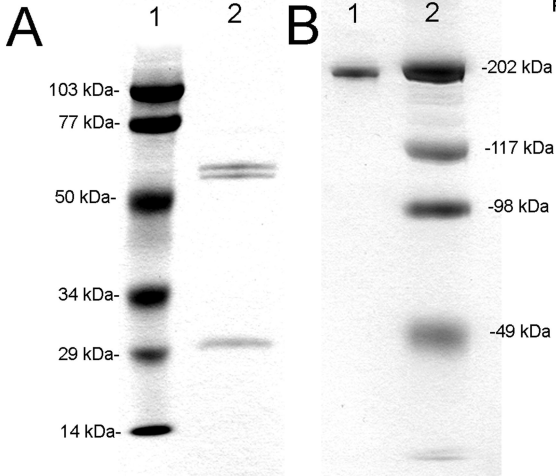


Figure 4

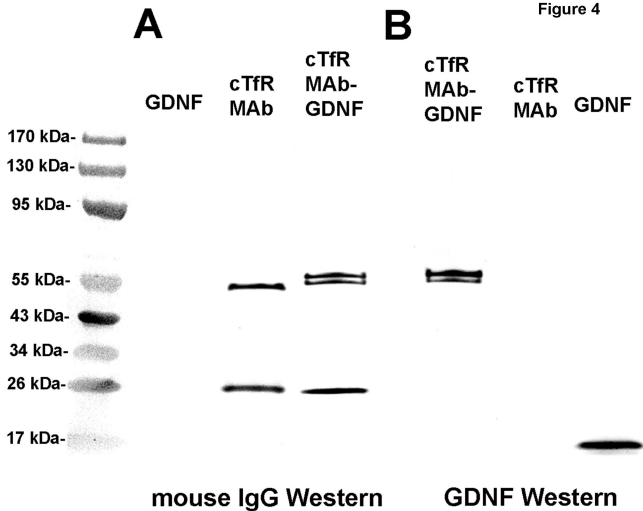
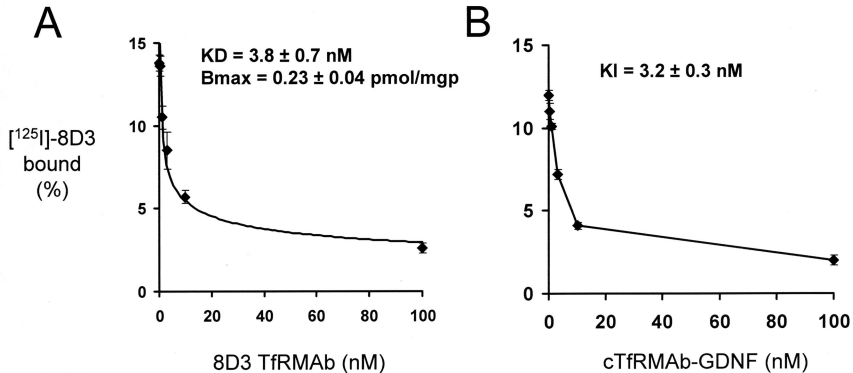


Figure 5



cTfRMAb-GDNF
ED₅₀ = 1.7 ± 0.2 nM

A405

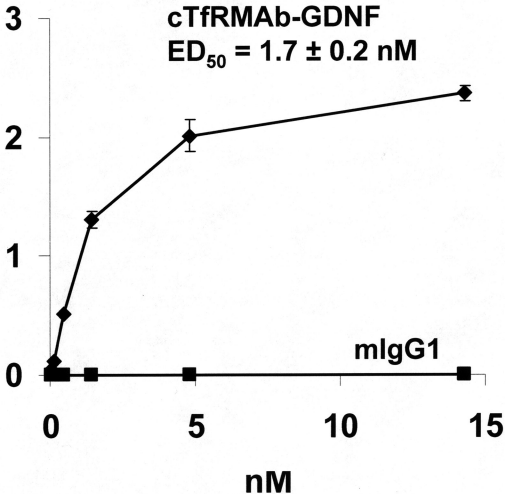


Figure 6

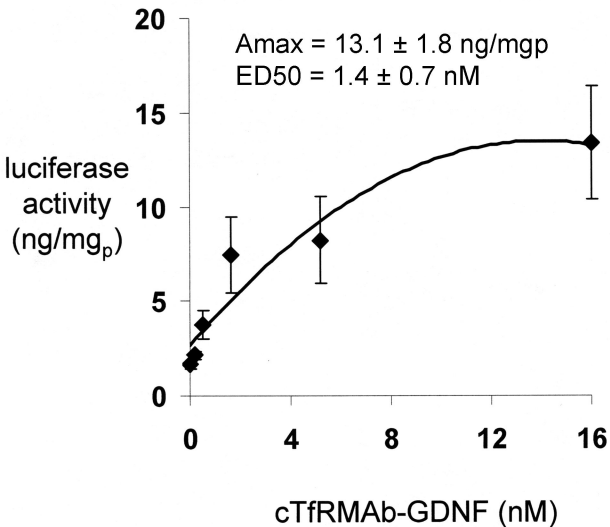


Figure 7

Figure 8

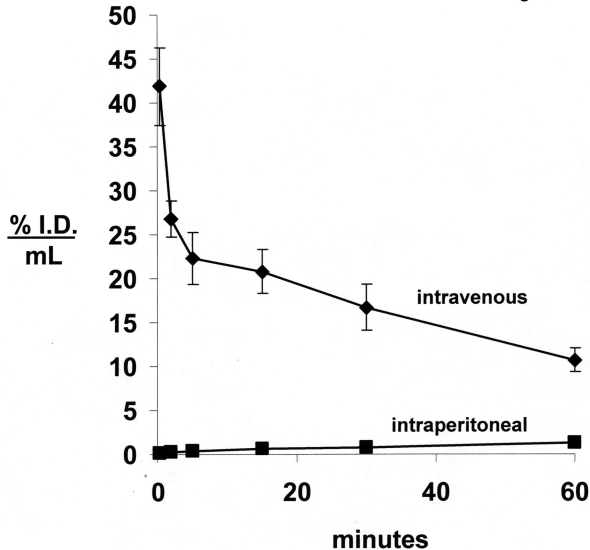


Figure 9

