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Chronic dosing of mice with a transferrin receptor monoclonal antibody-GDNF fusion protein

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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; CHO, Chinese hamster ovary; RT, room temperature; TCA, trichloroacetic acid; PK, pharmacokinetics; PS, permeability-surface area; AUC, area under the concentration curve; TIBC, total iron binding capacity.

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Abstract

Glial-derived neurotrophic factor (GDNF) is a potential neurotrophic factor treatment of brain disorders, including Parkinson's disease. However, GDNF does not cross the blood-brain barrier (BBB). A brain-penetrating form of GDNF has been engineered for the mouse, which is a fusion protein of human GDNF and a chimeric monoclonal antibody (MAb) against the mouse transferrin receptor (TfR), which is designated the cTfRMAb-GDNF fusion protein. The present study examines the potential toxic side effects and immune response following treatment of mice with twice-weekly cTfRMAb-GDNF fusion protein at a dose of 2 mg/kg IV for 12 consecutive weeks. Chronic treatment with the fusion protein caused no change in body weight, no change in 21 serum chemistry measurements, and no change in histology in brain and cerebellum, kidney, liver, spleen, heart, or pancreas. Chronic treatment caused a low titer immune response against the fusion protein, which was directed against the variable region of the antibody part of the fusion protein, with no immune response directed against either the constant region of the antibody, or against GDNF. A pharmacokinetics and brain uptake study was performed at the end of the 12 weeks of treatment. There was no change in clearance of the fusion protein mediated by the TfR in peripheral organs, and there was no change in BBB permeability to the fusion protein mediated by the TfR at the BBB. The study shows no toxic side effects from chronic cTfRMAb-GDNF systemic treatment, and the absence of neutralizing antibodies in vivo.

Introduction

Glial derived neurotrophic factor (GDNF) is a potential treatment for Parkinson's disease (PD), as GDNF is a trophic factor for the nigral-striatal tract in brain. However, GDNF does not cross the blood-brain barrier (BBB) (Kastin et al, 2003; Boado and Pardridge, 2009). GDNF can be made transportable through the BBB via receptor-mediated transport on an endogenous BBB peptide receptor following the re-engineering of the neurotrophin as an IgG-GDNF fusion protein. The IgG part of the fusion protein is a peptidomimetic monoclonal antibody (MAb) against an endogenous BBB receptor such as the insulin receptor or the transferrin receptor (TfR). The anti-receptor MAb binds an exofacial epitope on the BBB receptor, which triggers transport across the BBB, and acts as a molecular Trojan horse (MTH) to ferry into brain the fused GDNF (Boado and Pardridge, 2009). For drug delivery to the human brain, GDNF was fused to a genetically engineered MAb against the human insulin receptor (HIR) (Boado et al, 2008). However, the HIRMAb-GDNF fusion protein only cross-reacts with the insulin receptor in Old World primates such as the Rhesus monkey (Pardridge et al, 1995), and cannot be tested in rodent models. There is no known MAb against the rodent insulin receptor that can be used as a MTH in rats or mice. Therefore, a surrogate MTH for the mouse was engineered, which is a chimeric MAb against the mouse TfR, and designated the cTfRMAb (Boado et al, 2009). A fusion protein of the cTfRMAb and GDNF has been engineered, and is designated the cTfRMAb-GDNF fusion protein. The cTfRMAb-GDNF fusion protein is a bi-functional protein and binds both to the mouse TfR and to the GDNF receptor (GFR)-α1 with high affinity and low nM KD (Zhou et al, 2010). The cTfRMAb-GDNF fusion protein is rapidly transported

across the mouse BBB, and the in vivo brain uptake is 3.1% of injected dose (ID)/gram brain (Zhou et al, 2010). Chronic treatment of mice with experimental PD with intravenous (IV) cTfRMAb-GDNF fusion protein at a dose of 1 mg/kg every other day leads to a 272% increase in striatal tyrosine hydroxylase (TH) enzyme activity, and an improvement in neural deficit (Fu et al, 2010). However, the potential toxic effects of chronic administration of the cTfRMAb-GDNF fusion protein are not known. In addition, chronic administration of the cTfRMAb-GDNF fusion protein may lead to an immune response, and the formation of TfR neutralizing antibodies (NAb) could impair the biologic efficacy of the fusion protein in chronic treatment. Therefore, the purpose of the present study was the chronic dosing of mice with twice/weekly IV saline vehicle or cTfRMAb-GDNF fusion protein at a dose of 2 mg/kg/dose, or 4 mg/kg/week, for 12 consecutive weeks. To investigate potential toxicity, histology was examined on the brain and major peripheral organs and a panel of 21 serum chemistry parameters was analyzed in the saline and cTfRMAb-GDNF fusion protein treatment groups. The immune response was analyzed with a bridging ELISA, and a potential anti-TfR NAb response was evaluated by measurement of the plasma pharmacokinetics and brain uptake of the cTfRMAb-GDNF fusion protein at the end of the 12 week treatment period. Clearance of the fusion protein by peripheral organs was used as an index of potential neutralization of the peripheral TfR, and clearance of the fusion protein by brain was used as an index of potential neutralization of the TfR at the BBB.

Methods

Production of cTfRMAb-GDNF fusion protein fusion protein. The cTfRMAb-GDNF fusion protein was purified by protein G affinity chromatography of serum free medium conditioned by a stably transfected Chinese hamster ovary (CHO) line, as described previously (Zhou et al, 2010). The purity, identity, and potency of the fusion protein was verified by SDS-PAGE, mouse IgG and GDNF Western blotting, TfR radio-receptor assay and GFRα1 binding assay, as described previously (Zhou et al, 2010).

Chronic dosing of mice. Adult C57BL/6J mice, 10-12 weeks of age, were obtained from Jackson Labs (Bar Harbor, ME). The treatment group included 12 males, 28 gram body weight, and 12 females, 20 gram body weight. Mice were treated twice/week with a tail vein injection of 2 mg/kg (60 uL/mouse of 1 mg/mL) of cTfRMAb-GDNF fusion protein, or 60 uL/mouse of fusion protein vehicle (tris buffered saline, pH=5.5). Over 500 tail vein injections were performed for the study. After 12 weeks of treatment, mice were euthanized under anesthesia by cervical dislocation, and organs removed for histology and processed in 3 separate vials for fixation: (a) the entire cerebral hemisphere with cerebellum, (b) the heart, kidney, and liver, and (c) the spleen and pancreas. After 48 hours fixation in 10% buffered formalin, the tissues were embedded in paraffin and 5 micron sections were prepared for hematoxylin and eosin staining at the UCLA Translational Pathology Core Laboratory. The terminal serum was collected, frozen, and a Comprehensive Metabolic Panel, and an Anemia Panel (iron; total iron binding capacity) were analyzed at Molecular Diagnostic Services, Inc. (San Diego, CA).

Pharmacokinetics and brain uptake in the mouse. The cTfRMAb-GDNF fusion protein was tritiated with [3H]-N-succinimidyl propionate (American Radiolabeled Chemicals, Inc., St. Louis, MO) as described previously (Zhou et al, 2010). The specific activity was 0.6 uCi/ug and the trichloroacetic acid (TCA) precipitability was 95.5%. At the end of the 12-week dosing with either saline or fusion protein, 4 mice (2 males, 2 females) from the saline treatment group, and 4 mice (2 males, 2 females) from the cTfRMAb-GDNF fusion protein treatment group were tested for plasma clearance and brain uptake of the [^{3}H]-cTfRMAb-GDNF fusion protein, as described previously (Zhou et al, 2010). Mice were anesthetized with intra-peritoneal (IP) ketamine (100 mg/kg) and xylazine (10 mg/kg), and injected IV in the tail vein with 0.1 mL (10 uCi) of [^{3}H]-cTfRMAb-GDNF fusion protein. The injection dose in each mouse of the cTfRMAb-GDNF fusion protein was 0.8 mg/kg. An aliquot (50 uL) of heparinized blood was collected from the retro-orbital vein at 0.25, 2, 5, 15, 30, and 60 min from each mouse after injection of the fusion protein. The blood was centrifuged for collection of plasma, which was analyzed for radioactivity. At 60 min after injection, the mice were euthanized without saline perfusion of organs, and major organs, and the cerebral hemispheres were removed, weighed, and solubilized in Soluene-350 (Perkin Elmer, Downers Grove, IL), and analyzed for ^{3}H radioactivity with Optifluor-O (Perkin Elmer) and a liquid scintillation counter (Tricarb 2100TR, Perkin Elmer). Brain uptake data was expressed as the % of injected dose (ID)/gram tissue.

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

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

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The intercepts (A1, A2) and the slopes (k1, k2) were used to compute the pharmacokinetic (PK) parameters, including the mean 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 (Statistical Solutions Ltd, Cork, Ireland). Data were weighted by $1/(\%ID/mL)^2$.

The brain clearance (μL/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:

$$PS\ product = [(\%ID/g)/AUC]x1000$$

The brain uptake, or %ID/g, was first corrected by the brain uptake in the mouse of an IgG confined to the brain vascular volume, which is 0.06% ID/g (Zhou et al, 2010).

Immunity ELISA. The presence of anti-cTfRMAb-GDNF fusion protein antibodies in mouse serum was detected with a bridging ELISA, using the cTfRMAb-GDNF fusion protein as the capture reagent and biotinylated cTfRMAb-GDNF fusion protein as the detector reagent. Alternatively, the CHO cell derived cTfRMAb (Boado et al, 2009), mouse IgG1κ, which is the isotype antibody for the constant regions of the fusion protein (Sigmal Chemical Co., St. Louis, MO), the rat 8D3 mAb against the mouse TfR, which has the same variable regions as the fusion protein (Lee et al, 2000), or human recombinant GDNF (Peprotech, Rocky Hill, NJ) were used as the capture reagent. The mouse serum was diluted in PBS. The capture reagent was plated overnight at 4 °C in 96 wells at 100 μL (250 ng)/well in 0.05 M $NaHCO_3$/8.3. The wells were blocked with PBS containing 1% bovine serum albumin (PBSB), followed by the

addition of 100 µL/well of the diluted mouse serum. After a 60 min incubation at 37 °C, the wells were washed with PBSB, and the wells were incubated with biotinylated cTfRMAb- GDNF fusion protein (12 ng/well) for 60 min. The wells were washed with PBSB, followed by incubation with 100 µL (500 ng/well) of a streptavidin−peroxidase conjugate (#SA-5004, Vector Laboratories) for 30 min at RT. The wells were washed with PBSB, and 100 µL/well of *o*-phenylenediamine/H_2O_2 developing solution (#P5412, Sigma) was added for a 15 min incubation in the dark at RT. The reaction was stopped by the addition of 100 µL/well of 1 M HCl, followed by the measurement of absorbance at 492 and 650 nm. The A650 was subtracted from the A492. The (A492 − A650) for the PBSB blank was then subtracted from the (A492 − A650) for the sample. Mouse serum samples were screened with the immunity ELISA at 1:50 dilutions in PBS using the cTfRMAb-GDNF fusion protein as the capture reagent. For subsequent studies, and since the immunoreactivity was comparable in all fusion protein treated mice, the terminal serum from all mice treated with the cTfRMAb-GDNF fusion protein was pooled. This pool was then diluted 1:50, 1:100 1:300, 1:1000, or 1:3000 in PBS. A mouse monoclonal GDNF-neutralizing antibody (R&D Systems, Minneapolis, MN) was tested at concentrations ranging from 0.1 to 30 ug/mL, and was used as a positive control in the assay for detection of anti-GDNF antibodies in the mouse serum. The mouse dilution curves were determined for different capture reagents: the CHO-derived cTfRMAb, the hybridoma-derived rat 8D3 mAb against the mouse TfR, GDNF, or mouse IgG1k, which is the isotype control for the constant region comprising the cTfRMAb. The cTfRMAb-GDNF fusion protein was biotinylated as described previously (Pardridge and Boado, 2009), using sulfo-biotin-LC-LC-*N*-hydroxysuccinimide, where

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LC = long chain (#21338, Pierce Chemical Co., Rockford, IL). The biotinylation of the cTfRMAb-GDNF fusion protein was confirmed by SDS−PAGE and Western blotting, where the blot was probed with avidin and biotinylated peroxidase. The nonbiotinylated cTfRMAb-GDNF fusion protein gave no reaction in the Western blot, whereas the biotinylated protein was strongly visualized at the appropriate molecular size for both heavy chain and light chain.

Statistics. Statistical differences at the $p<0.05$ level were determined by Student's t-test.

Results

All 24 mice tolerated well the chronic treatment with twice-weekly cTfRMAb-GDNF fusion protein or saline via tail vein injection. There was no difference in body weights between the males or females of the saline or fusion protein-treated groups (Table 1). No mice exhibited any clinical signs of immune reactions to the fusion protein, and no mice required treatment with diphenhydramine or other immune response modifiers. There was no difference in 23 serum chemistries between the saline and fusion protein treated mice, including no differences in serum iron or total iron binding capacity (TIBC) (Table 2). No pathologic findings were observed in brain in any mice after review of sagittal sections encompassing the olfactory lobe to the cerebellum. Layers of the cerebellum, including the granular layer, the Purkinje cell layer, and the molecular layer showed normal histology (Figure 1A). Purkinje cell dendrites were visible in the molecular layer in the fusion protein treated mice to the same extent as in the saline treated mice. No abnormalities were observed in peripheral organs (liver, spleen, heart, kidney, and pancreas), and representative organ histology is shown in Figure 1 for the fusion protein treated mice.

The design of the immunity bridging ELISA is shown in Figure 2A; owing to antibody bivalency, the anti-fusion protein antibodies in mouse serum bind both the capture reagent and the biotinylated fusion protein detector reagent. There was time-dependent increase in immune response directed against the cTfRMAb-GDNF fusion protein over the course of the 12 week treatment period in all fusion protein treated mice (Figure 2B). The absorbance readings at 2, 4 and 12 weeks were averaged and compared to the mean absorbance readings in the saline treated mice, which showed

no immune response against the fusion protein in the saline treated mice (Figure 2C). The absorbance readings shown in Figure 2 were all determined with 1:50 dilutions of mouse sera. In order to determine the titer of the immune response against different portions of the fusion protein, the serum of all fusion protein treated mice collected after 12 weeks of treatment was pooled and diluted from 1:50 to 1:3000. When the cTfRMAb-GDNF fusion protein was used as the capture reagent, the absorbance was near background at a 1:1000 dilution (Figure 3A). The anti-fusion protein antibodies in the 12 week mouse serum pool also reacted with the original rat 8D3 TfRMAb and the cTfRMAb, but there was minimal reaction against GDNF (Figure 3A). Mouse IgG1k is the isotype antibody for the constant region of the heavy and light chains of the fusion protein. When mouse IgG1k was used as the capture reagent, there was no immune reaction detected. So as to demonstrate the bridging ELISA outlined in Figure 2A could detect antibodies against the GDNF portion of the fusion protein, a mouse MAb against human GDNF was assayed. As shown in Figure 3B, there is a dose-dependent and saturable immunoreactivity of this antibody in the immunity ELISA.

Any anti-TfR neutralizing antibodies (NAb) in the blood of the fusion protein mice could potentially block fusion protein binding to the TfR in either peripheral organs or at the BBB. To determine if any anti-TfR NAb's are formed, the [^{3}H]-cTfRMAb-GDNF fusion protein was injected IV in 4 of the fusion protein treated mice (2 males; 2 females) and 4 of the saline treated mice (2 males; 2 females) prior to euthanasia at the end of the 12 week treatment period. There is no change in the rate of removal of the fusion protein from blood via clearance by peripheral organs (Figure 4). The fusion protein was metabolically stable in both treatment groups, as the plasma radioactivity at

60 min after IV injection was 95 ± 2% in both groups. There is no change in the plasma pharmacokinetic parameters in the saline-treated and fusion protein-treated mice (Table 3). There is no change in uptake of the fusion protein by brain or peripheral organs in the saline-treated and fusion protein-treated mice (Table 4). The brain uptake, %ID/gram (Table 4), and the 60 min plasma AUC (Table 3), were used to compute the BBB PS product of fusion protein, and there was no change in BBB transport of the cTfRMAb-GDNF fusion protein in the saline-treated and fusion protein-treated mice (Figure 5).

Discussion

The findings of this study are consistent with the following conclusions. First, chronic treatment of mice with IV cTfRMAb-GDNF fusion protein causes no toxic side effects, as there is no change in body weight (Table 1), no change in serum chemistry (Table 2), and no change in organ histology (Figure 1). Second, chronic treatment with the fusion protein induces a time-dependent immune response (Figure 2), which is low titer and directed against the variable region of the cTfRMAb part of the fusion protein (Figure 3). Third, the antibodies formed against the cTfRMAb have no functional effect, as the rate of clearance of the fusion protein mediated by the TfR in peripheral organs is unchanged (Figure 4, Tables 3-4), and the clearance of the fusion protein by brain mediated by the BBB TfR is unchanged (Figure 5).

The biological effects of GDNF, and related neurotrophins (persephin, neurturin, artemin), are mediated by binding of the neurotrophin to the cognate receptor, which for GDNF is GFRα1. Receptor binding then triggers activation of the c-ret kinase within the target cell (Airaksinen and Saarma, 2002). GDNF, GFRα1, and the c-ret kinase are expressed in peripheral organs, as well as the CNS. In the mouse, GFRα1 mRNA is highly expressed in peripheral nerve, liver, and kidney, whereas the c-ret kinase mRNA is highly expressed in peripheral nerve, pituitary, heart, and skeletal muscle (Naveilhan et al, 1998). GDNF may have a role in development of the kidney (Vega et al, 1996) and the pancreas (Lucini et al, 2008). GFRα1 and c-ret are expressed in the heart, and play a role in the cholinergic innervation of the heart (Hiltunen et al, 2000). There was no change in body weight (Table 1), organ histology in kidney, liver, spleen, heart, or pancreas (Figure 1), and there was no change in 23 serum chemistries that reflect

hepatic, renal, metabolic, and iron function (Table 2). The TfRMAb part of the cTfRMAb-GDNF fusion protein may potentially have effects on iron homeostasis. However, chronic treatment with the fusion protein has no effect on serum levels of iron or total iron binding capacity (TIBC) (Table 2).

The chronic infusion in the brain of high doses of GDNF for 6 months in the Rhesus monkey led to cerebellar degeneration (Hovland et al, 2007). However, in the present study, there was no evidence of toxicity in brain following 12 weeks of twice-weekly intravenous injections of the cTfRMAb-GDNF fusion protein (Figure 1). There is no cerebellar degeneration in the fusion protein treated mice, and the granule cell layer, the Purkinje cell layer, and the molecular layer of the cerebellum in the fusion protein treated mice were indistinguishable from that of the saline treated mice (Figure 1A).

The fusion protein treated mice developed a time-dependent immune response following 12 weeks of intravenous treatment (Figure 2). However, the development of an immune response in the chronic treatment with a biologic is expected. What is important is the titer of the immune response and whether the antibodies formed against the fusion protein neutralize therapeutic action in vivo. The titer of the immune response is quantitated as the OD units per uL undiluted serum (Dickson et al, 2008). A titer of <10 is considered evidence of tolerance to the biologic agent (Dickson et al, 2008). The immunity ELISA records 1.5 OD units per 100 uL of a 1:50 dilution of the mouse serum (Figure 2), which is a titer of 0.75 OD/uL. The low titer of the immune response against the cTfRMAb-GDNF fusion protein is also demonstrated with the dilution curve (Figure 3), which shows 0.09 OD units at a dilution of 1:1000, which corresponds to a titer of 0.9 OD/uL.

The use of different capture reagents in the immunity ELISA allows for identification of the domain of the cTfRMAb-GDNF fusion protein that accounts for the majority of the immune reactions against the fusion protein (Figure 3). The fusion protein is comprised of 3 domains: the variable regions of the heavy chain (VH) and the light chain (VL), which arise from a rat IgG against the murine TfR (Boado et al, 2009), the heavy chain and light chain constant regions, which are derived from mouse IgG1 and mouse kappa, respectively (Boado et al, 2009), and human GDNF (Zhou et al, 2010). The immune response against the GDNF part of the fusion protein is negligible (Figure 3A). So as to confirm the immunity ELISA could detect antibodies against the GDNF part of the fusion protein, a mouse neutralizing anti-GDNF antibody was studied, and this antibody reacted strongly in the immunity ELISA (Figure 3B). In contrast to the minimal immune response against the GDNF part of the IgG-GDNF fusion protein in the present study, a peripheral immune response against GDNF was observed following the chronic infusion of GDNF into the brain of either Rhesus monkeys (Hovland et al, 2007) or humans (Tatarewicz et al, 2007). The absence of a stronger immune response against the GDNF part of the cTfRMAb-GDNF fusion protein in the present study may be related to the presence of certain amino acid sequences, called Tregitopes, within the IgG constant region, which induce immune tolerance (DeGroot et al, 2008).

The immune response against the cTfRMAb-GDNF fusion protein is primarily directed against the variable region of the cTfRMAb (Figure 3). The variable region is comprised of the framework regions and the complementarity determining regions (CDR) of the antibody. If antibodies are formed against the CDR of the cTfRMAb, these could potentially neutralize antibody function in vivo by blocking cTfRMAb binding to the

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TfR. Neutralizing antibody assays are typically performed with cell-based bioassays in vitro. However, such an assay may not predict the process of receptor-mediated transport across the BBB in vivo via transport on the endogenous TfR. Therefore, in the present study, the pharmacokinetics (PK) and brain uptake of the [^{3}H]-cTfRMAb-GDNF fusion protein was assessed at the end of the 12-week treatment study in 4 mice from the saline treated group and 4 mice from the fusion protein treated group. The rate of clearance of the fusion protein from blood (Figure 4), the PK parameters (Table 3), and the uptake of the fusion protein by peripheral tissues (Table 4), was unchanged in the two treatment groups. These findings indicate there is no neutralization of the uptake of the cTfRMAb-GDNF fusion protein via the TfR in peripheral organs. Similarly, there is no change in the brain uptake of the fusion protein (Table 4), or the BBB permeability of the fusion protein (Figure 5) in the mice treated chronically with the cTfRMAb-GDNF fusion protein fusion protein. Therefore, there is no neutralization of the transport of the fusion protein via the BBB TfR in vivo.

In summary, chronic administration of the cTfRMAb-GDNF fusion protein in mice is shown to have a favorable safety profile with no histologic abnormalities in brain or peripheral organs, and no change in serum chemistry. The immune response against the fusion protein generated by chronic intravenous treatment in the mouse is low titer, and has no functional consequences on the distribution of the fusion protein in brain in vivo.

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Authorship Contributions

Participated in research design: Zhou, Boado, Lu, Hui, Pardridge

Conducted experiments: Zhou, Boado, Lu, Hui, Pardridge

Performed data analysis: Zhou, Boado, Lu, Hui, Pardridge

Wrote or contributed to the writing of the manuscript: Zhou, Boado, Lu, Hui, Pardridge

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Footnotes

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Legends to Figures

Figure 1. Hematoxylin and eosin histology for cerebellum (A), kidney (B), liver (C), spleen (D), heart (E), and pancreas (F). In the cerebellum, Purkinje cells are observed at the interface of the granular layer (top) and the molecular layer (bottom) of the section. Magnification is the same in panels B-F. Magnification bars in panels A and panel and B are 42 and 210 microns, respectively.

Figure 2. (A) Structure of the bridging ELISA for detection of antibodies against the cTfRMAb-GDNF fusion protein. The cTfRMAb- GDNF fusion protein is used as the capture reagent, and the biotinylated cTfRMAb- GDNF fusion protein is used as the detector reagent, along with a complex of streptavidin (SA) and horseradish peroxidase (HRP); the biotin moiety is designated “B”. (B) The immune response in the individual mice of the fusion protein treated mice is plotted against the number of weeks of treatment. (C) The mean immune response in the mice treated with either fusion protein or saline is plotted against the number of weeks of treatment. The capture reagent in the assays shown in panels B and C was the cTfRMAb-GDNF fusion protein.

Figure 3. (A) The terminal 12-week serum from all fusion protein treated mice were pooled and diluted 1:50 to 1:3000 in PBS, and immunoreactivity was measured against 4 different capture reagents: cTfRMAb, 8D3 TfRMAb, the cTfRMAb-GDNF fusion protein, and GDNF. (B) The immunoreactivity of a mouse anti-GDNF antibody is plotted against the antibody concentration; the capture reagent in this assay was the cTfRMAb-GDNF fusion protein.

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Figure 4. Plasma concentration, expressed as percentage of ID/ml, of the [^{3}H]cTfRMAb-GDNF fusion protein after intravenous injection in mice from either the saline treatment group or the cTfRMAb-GDNF fusion protein. Males and females are combined, as there were no differences between sexes. Data are mean ± S.E. (n = 4 mice/point).

Figure 5. BBB permeability-surface area (PS) produce of the [^{3}H]cTfRMAb-GDNF fusion protein measured in either the saline treatment group or the cTfRMAb-GDNF fusion protein group.

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Table 1. Body weights (grams)

weeks	cTfRMAb-GDNF		saline	
	Male	**Female**	**Male**	**Female**
0	28.1 ± 2.1	20.2 ± 1.0	29.0 ± 1.2	19.5 ± 1.6
3	28.4 ± 1.9	20.4 ± 1.2	28.9 ± 0.9	21.1 ± 2.0
6	29.6 ± 1.3	22.4 ± 0.8	31.1 ± 0.9	22.4 ± 1.9
9	30.4 ± 1.5	22.6 ± 0.8	32.9 ± 0.7	22.7 ± 2.3
12	31.3 ± 2.0	23.4 ± 1.2	33.4 ± 0.6	23.5 ± 2.5

Mean ±SD (n= 6 mice in each of the 4 treatment groups).

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Table 2. Serum metabolic panel

parameter	units	Treatment group	
		saline	cTfRMAb-GDNF
Sodium	mEq/L	151 ±2	151 ±2
Potassium	mEq/L	4.8 ± 0.5	5.1 ± 0.5
Chloride	mEq/L	125 ± 6	124 ± 5
CO2	mEq/L	24 ± 4	23 ± 3
Calcium	mg/dL	9.7 ± 0.3	10.2 ± 0.3
Phosphorous	mg/dL	8.9 ± 0.7	9.5 ± 1.4
Magnesium	mg/dL	4.4 ± 0.2	4.6 ± 0.1
Glucose	mg/dL	205 ± 35	213 ± 38
BUN	mg/dL	22 ± 1	26 ± 3
Creatinine	mg/dL	0.3 ± 0.1	0.3 ± 0.1
Total bilirubin	mg/dL	0.6 ± 0.3	0.7 ± 0.3
Direct bilirubin	mg/dL	<0.1	<0.1
Total protein	g/dL	4.8 ± 0.1	4.9 ± 0.3
Albumin	g/dL	3.1 ± 0.2	3.3 ± 0.1
Globulin	g/dL	1.7± 0.2	1.6± 0.3
Uric acid	mg/dL	2.4 ± 0.2	3.1 ± 0.8
AST	IU/mL	88 ± 24	98 ± 7
ALT	IU/mL	35 ± 10	31 ± 13
ALK	IU/mL	72 ± 26	77 ± 22
GGT	IU/mL	<2	<2
Creatine kinase	IU/mL	172 ± 71	280 ± 32
iron	ug/dL	128 ± 11	132 ± 8
TIBC	ug/dL	271 ± 18	278 ± 10

Mean ± SD (n=6 mice/group). No statistical differences between the 2 groups. Males and females are combined, as there were no differences between sexes.
AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALK=alkaline phosphatase; GGT=γ-glutamyl transpeptidase; BUN=blood urea nitrogen; IU=international unit; TIBC=total iron binding capacity.

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Table 3. Pharmacokinetic parameters

parameter	units	Treatment group	
		cTfRMAb-GDNF	saline
A1	%ID/mL	18.4 ± 3.4	21.4 ± 3.9
A2	%ID/mL	18.4 ± 1.3	16.2 ± 1.8
K1	min^{-1}	0.73 ± 0.25	0.38 ± 0.12
K2	min^{-1}	0.011 ± 0.002	0.0067 ± 0.0027
MRT	min	89 ± 15	146 ± 59
Vc	mL/kg	97 ± 9	95 ± 9
Vss	mL/kg	188 ± 12	210 ± 23
AUC (60 min)	%ID•min/mL	831 ± 27	859 ± 36
AUCss	%ID•min/mL	1681 ± 201	2479 ± 777
Cl	mL/min/kg	2.12 ± 0.25	1.44 ± 0.47

Mean ± SD. Males and females are combined, as there were no differences between sexes.

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Table 4. Organ uptake of cTfRMAb-GDNF fusion protein

Organ	Treatment group	
	cTfRMAb-GDNF	**saline**
Heart	2.00 ± 0.80	2.41 ± 0.70
Liver	9.76 ± 2.19	11.3 ± 3.4
Spleen	14.5 ± 3.7	13.0 ± 4.1
Lung	11.0 ± 3.5	10.4 ± 2.8
kidney	4.60 ± 0.94	3.46 ± 0.78
brain	2.54 ± 0.90	2.60 ± 0.61

Mean ± SD (n=4 per group). Males and females are combined, as there were no differences between sexes.

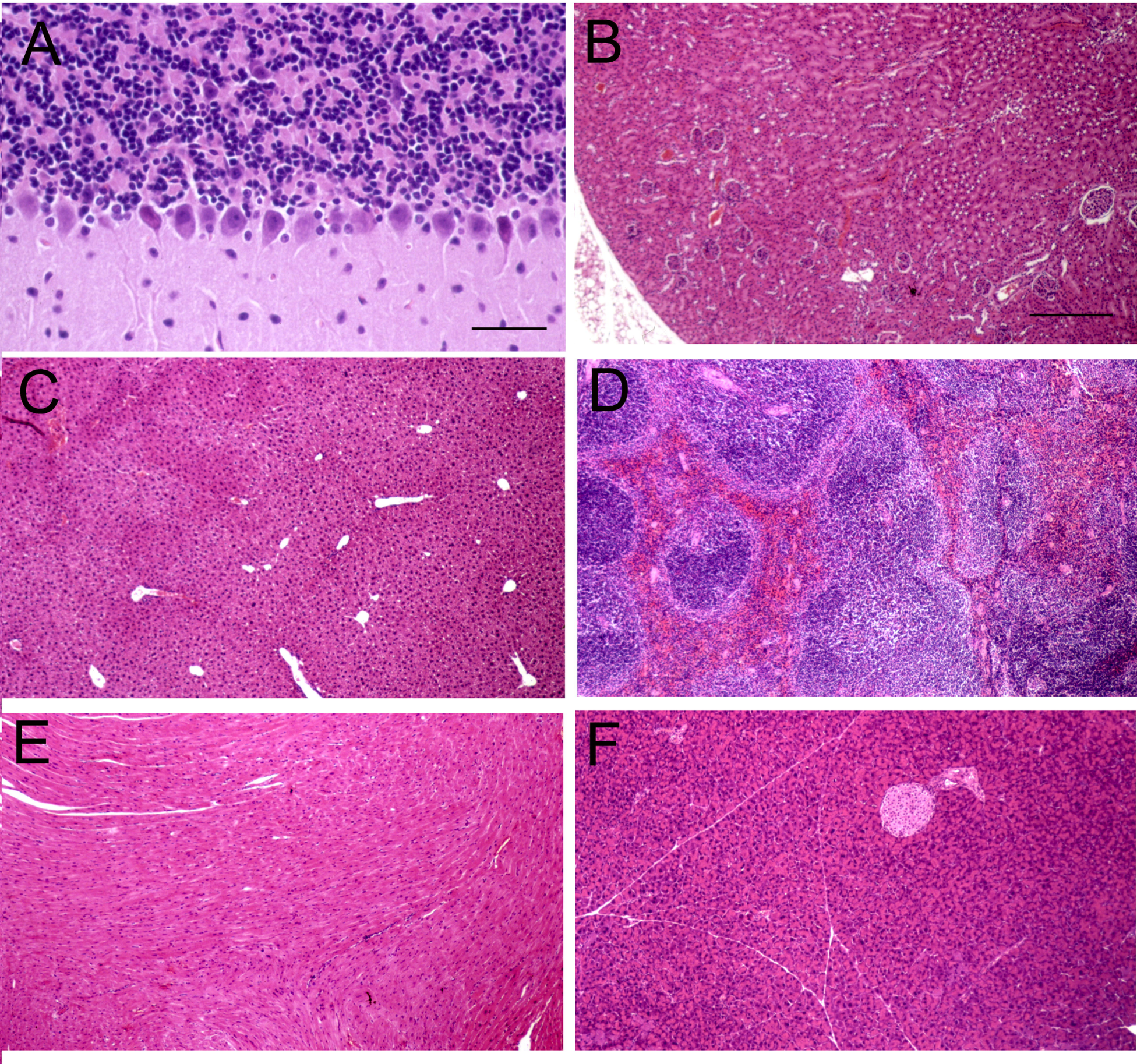


Figure 1

Figure 2

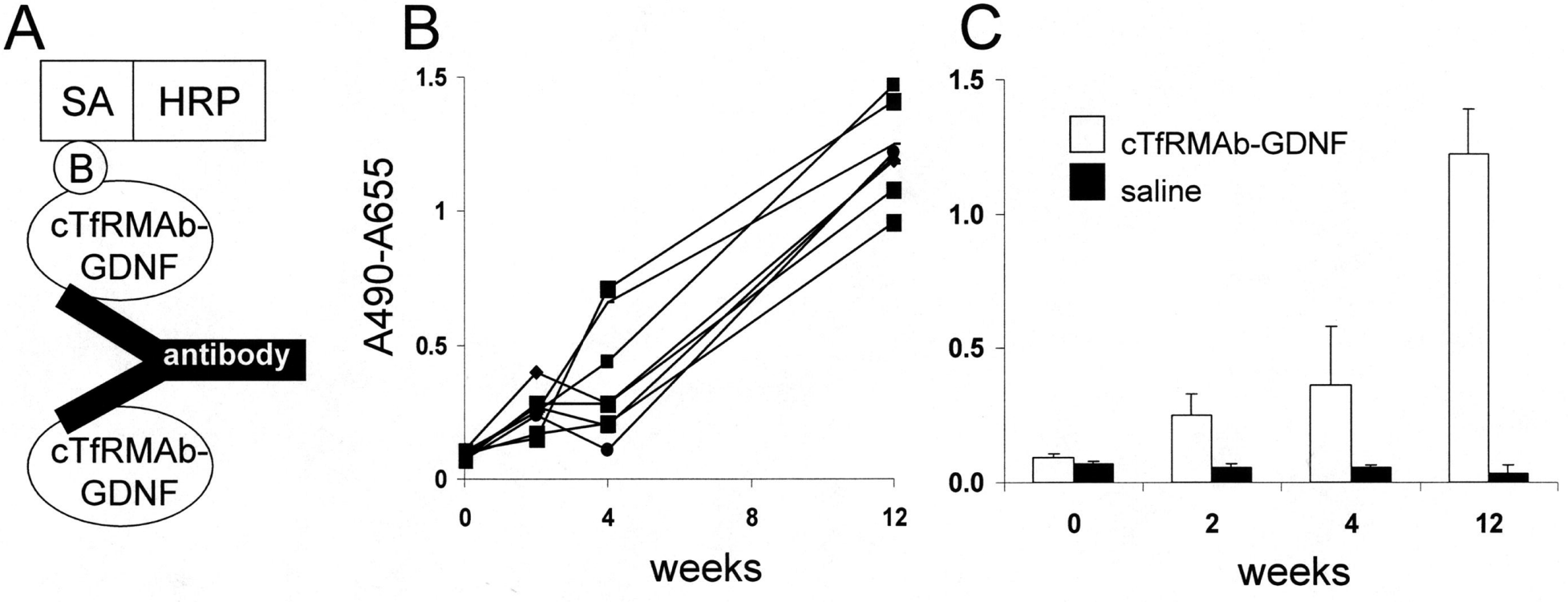
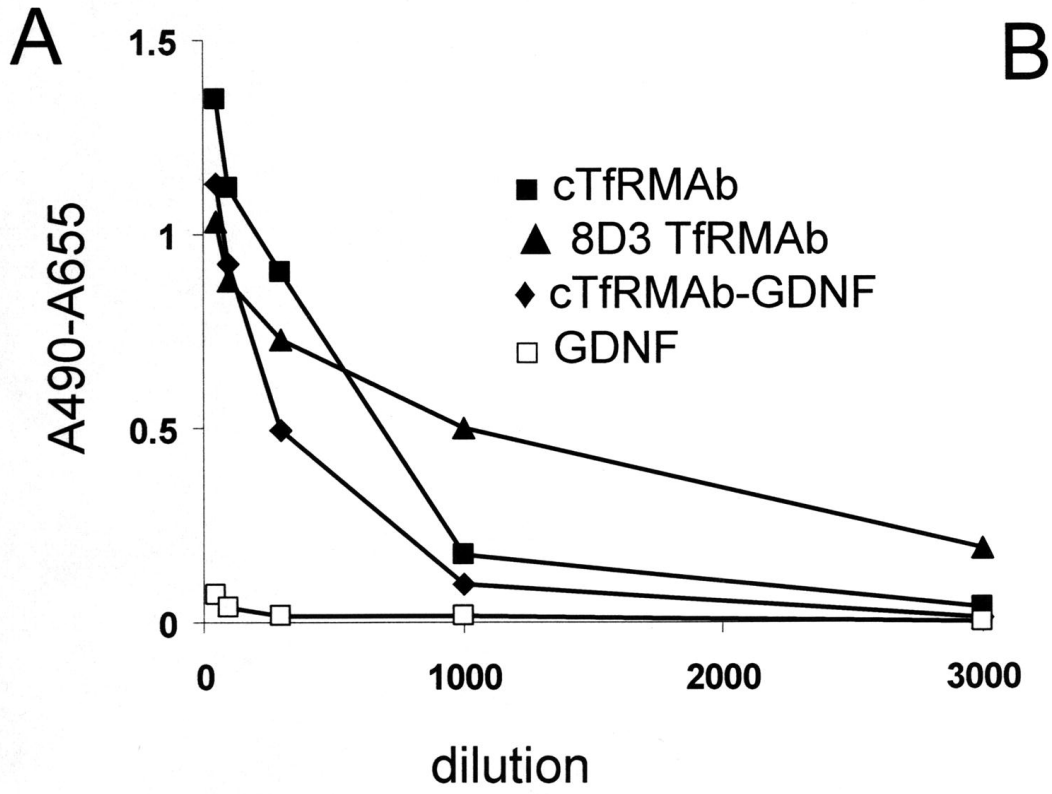

A
SA
HRP
B
cTfRMAb-GDNF
antibody
cTfRMAb-GDNF
B
A490-A655
1.5
1
0.5
0
0
4
8
12
weeks
C
1.5
1.0
0.5
0.0
cTfRMAb-GDNF
saline
0
2
4
12
weeks

Figure3


A
1.5
1
0.5
0
A490-A655
cTfRMAb
8D3 TfRMAb
cTfRMAb-GDNF
GDNF
0
1000
2000
3000
dilution

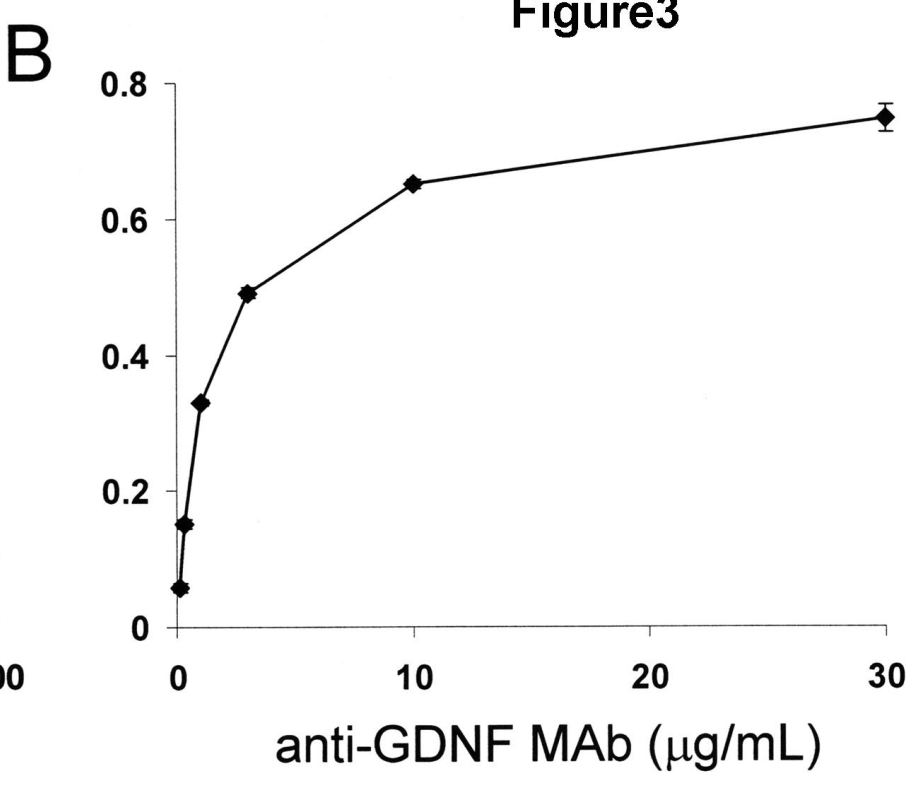

B
0.8
0.6
0.4
0.2
0
0
10
20
30
anti-GDNF MAb (μg/mL)

Figure 4

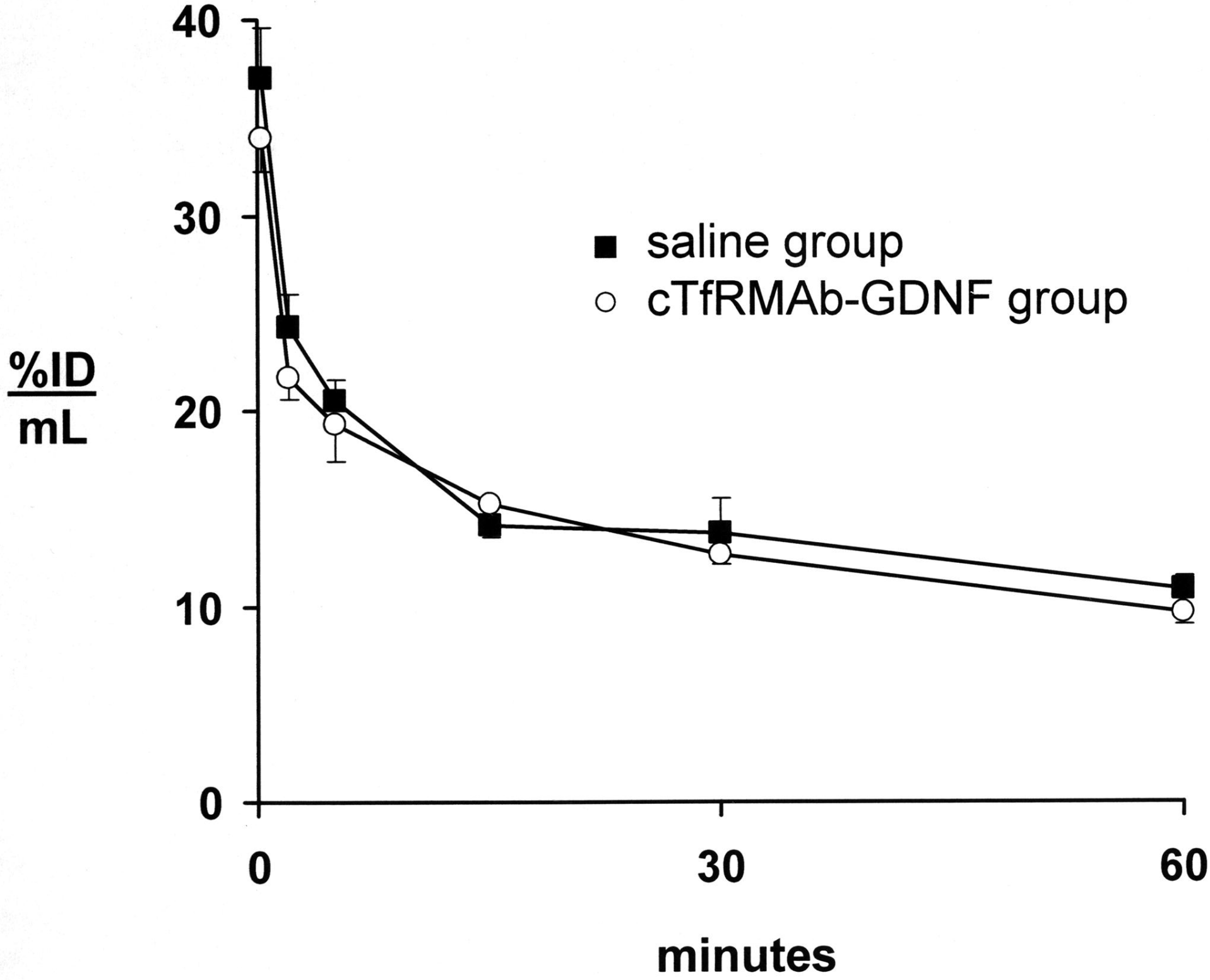
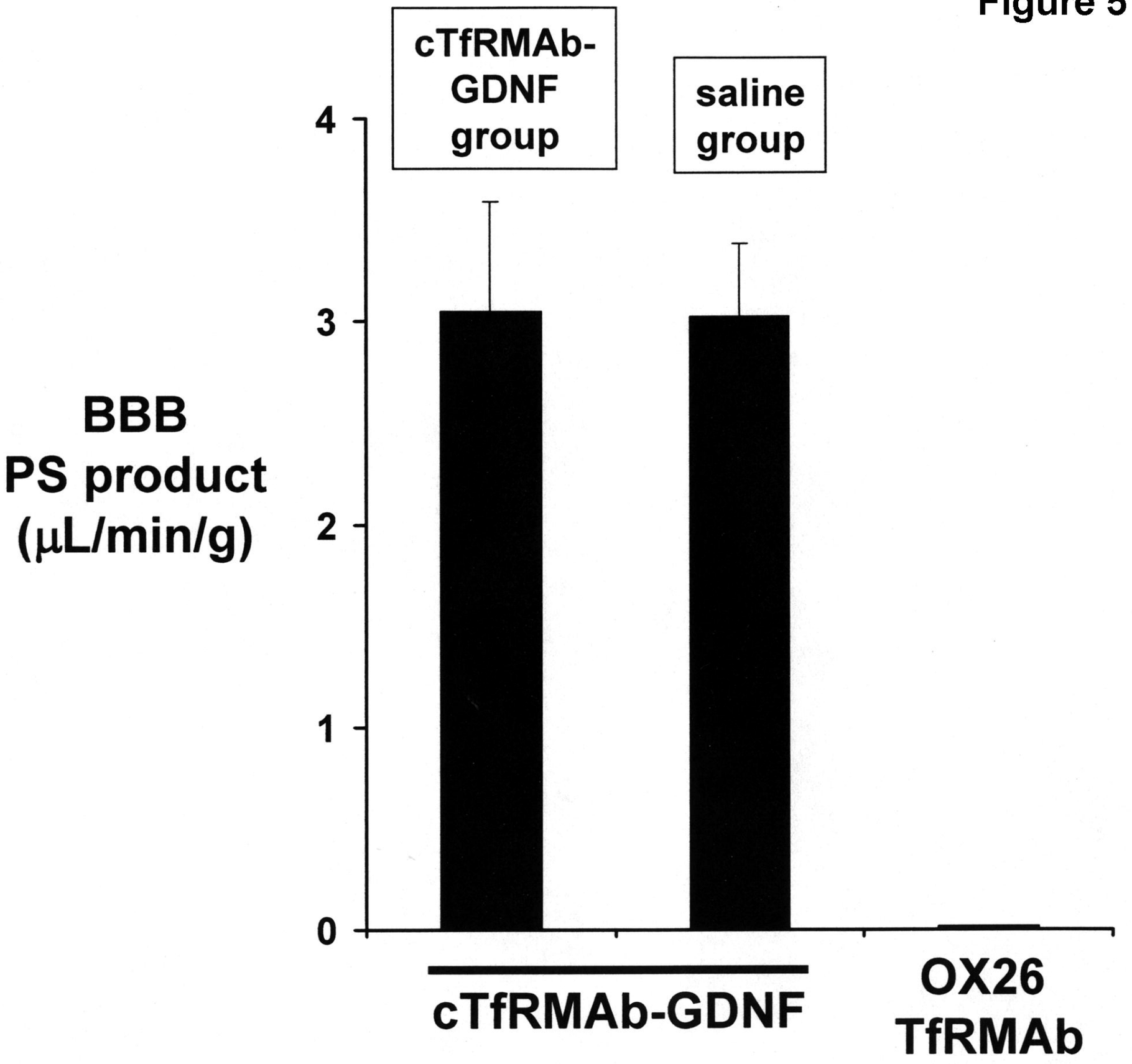

40
30
20
10
0
%ID
mL
saline group
cTfRMAb-GDNF group
0
30
60
minutes

Figure 5


cTfRMAb-GDNF group
saline group
4
3
2
1
0
BBB PS product (μL/min/g)
cTfRMAb-GDNF
OX26 TfRMAb