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Intravenous treatment of experimental Parkinson's disease in the mouse with an IgG-GDNF fusion protein that penetrates the blood-brain barrier

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Abstract

Glial derived neurotrophic factor (GDNF) is a trophic factor for the nigra-striatal tract in experimental Parkinson's disease (PD). The neurotrophin must be administered by intra-cerebral injection, because GDNF does not cross the blood-brain barrier (BBB). In the present study, GDNF was re-engineered to enable receptor-mediated transport across the BBB following fusion of GDNF to the heavy chain of a chimeric monoclonal antibody (MAb) against the mouse transferrin receptor (TfR), and this fusion protein is designated cTfRMAb-GDNF. This fusion protein had been previously shown to retain low nM binding constants for both the GDNF receptor and the mouse TfR, and to rapidly enter the mouse brain in vivo following intravenous administration. Experimental PD in mice was induced by the intra-striatal injection of 6-hydroxydopamine, and mice were treated with either saline or the cTfRMAb-GDNF fusion protein every other day for 3 weeks, starting 1 hour after toxin injection. Fusion protein treatment caused a 44% decrease in apomorphine-induced rotation, a 45% reduction in amphetamine-induced rotation, a 121% increase in the vibrissae-elicited forelimb placing test, and a 272% increase in striatal tyrosine hydroxylase (TH) enzyme activity at 3 weeks after toxin injection. Fusion protein treatment caused no change in TH enzyme activity in either the contralateral striatum or the frontal cortex. In conclusion, following fusion of GDNF to a BBB molecular Trojan horse, GDNF trophic effects in brain in experimental PD are observed following intravenous administration.

Keywords

blood-brain barrier; Parkinson's disease; GDNF; monoclonal antibody

1. Introduction

Glial-derived neurotrophic factor (GDNF) is a neurotrophin active in the nigral-striatal tract of the brain. The intra-cerebral injection of the GDNF recombinant protein (Hoffer et al, 1994; Lapchak et al, 1997) has both protective and rescue properties in experimental Parkinson's disease (PD). However, the bilateral trans-cranial infusion of GDNF into the

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putamen in human PD was not therapeutic (Lang et al, 2006). This failure of GDNF in human PD was shown to be due to poor penetration of GDNF into brain tissue following intra-cerebral infusion. A steep logarithmic decrease in the brain GDNF concentration was observed over a distance of just 6 mm removed from the infusion catheter in the primate brain, and it was estimated that only 2–9% of the GDNF infused directly into the brain penetrated the putamen (Salvatore et al, 2006).

The alternative approach to brain drug delivery of GDNF is the trans-vascular route across the blood-brain barrier (BBB). Owing to the short distances, 40 μm, between capillaries in the brain, drug distributes to all brain cells following delivery across the BBB (Pardridge, 2002). However, GDNF does not cross the BBB in either the mouse (Kastin et al, 2003) or the Rhesus monkey (Boado and Pardridge, 2009). Consequently, GDNF must be re-engineered to enable delivery across the BBB, and this is possible with molecular Trojan horse (MTH) technology (Pardridge, 2010). A MTH is an endogenous peptide or peptidomimetic monoclonal antibody (MAb) that is transported on an endogenous BBB receptor, such as the insulin receptor or the transferrin receptor (TfR). The most potent MTH known is a genetically engineered MAb against the human insulin receptor (HIR), and a fusion protein of the HIRMAb and human GDNF has been genetically engineered (Boado et al, 2008). The HIRMAb-GDNF fusion protein is a dual receptor-specific protein with low nM binding constants for both the HIR, to mediate delivery across the BBB, and the GDNF receptor (GFR)α1, to mediate GDNF trophic effects on neurons behind the BBB. However, the HIRMAb-GDNF fusion protein cannot be tested in rodent models of PD, because the HIRMAb does not cross react with the rodent insulin receptor (Pardridge et al, 1995), and there is no known MAb against the rodent insulin receptor that could be used as a MTH in the mouse or rat. Consequently, a surrogate MTH for use in the mouse has been engineered, which is a chimeric MAb against the mouse TfR, designated the cTfRMAb (Boado et al, 2009). Recently, a fusion protein of human GDNF and the cTfRMAb, designated the cTfRMAb-GDNF fusion protein, has been engineered and expressed in stably transfected Chinese hamster ovary (CHO) cells (Zhou et al, 2010). The cTfRMAb-GDNF fusion protein is bi-functional, and binds both the GFRα1 and the mouse TfR with low nM binding constants. The cTfRMAb-GDNF fusion protein is rapidly transported across the mouse BBB, and the brain uptake is 3.1 ± 0.2% of the injected dose (ID)/gram brain (Zhou et al, 2010). The present investigation is an extension of the prior study describing the cTfRMAb-GDNF fusion protein (Zhou et al, 2010), and was designed to test the therapeutic effects of chronic dosing in a murine model of PD. Experimental PD is produced in mice with the intra-cerebral injection of 6-hydroxydopamine (Lundblad et al, 2004). The therapeutic effect of fusion protein treatment was evaluated with 3 models of neurobehavior in the mice, as well as striatal tyrosine hydroxylase (TH) enzyme activity. TH was used as a GDNF-dependent striatal function, as TH gene expression is regulated by the GDNF receptor (GFR) linked c-ret kinase (Xiao et al, 2002).

2. Results

Experimental PD was induced in mice by the intra-striatal injection of 6-hydroxydopamine on day 1 of the study. The mice were then treated every other day with 1 mg/kg of cTfRMAb-GDNF fusion protein given intravenously (IV), starting 1 hour after toxin injection. Mice were tested for apomorphine-induced and amphetamine-induced rotation at 1, 2, and 3 weeks after toxin administration, and were then euthanized for measurement of striatal TH enzyme activity. The mice treated with saline showed an increase in apomporphine-induced contralateral rotation at 1 through 3 weeks after toxin injection (Figure 1). However, the mice treated with the cTfRMAb-GDNF fusion protein exhibited a 44% decrease in apomorphine-induced rotation at both 2 and 3 weeks after toxin administration. The mice treated with saline similarly showed an increase in amphetamine-

induced ipsilateral rotation at 1 through 3 weeks after toxin injection (Figure 2). However, the mice treated with the cTfRMAb-GDNF fusion protein demonstrated a 62% and 45% reduction in amphetamine-induced rotation behavior at 2 and 3 weeks, respectively, after toxin administration (Figure 2). At the end of the study, the mice were evaluated with the vibrissae-elicited forelimb placing test. The saline and fusion protein treated mice both showed maximal placing scores on the right side, which is ipsilateral to the side of toxin injection (Figure 3). The mice treated with saline showed a reduction in placing score from 169 ± 2, on the non-lesioned side, to 34 ± 4, on the lesioned side (mean ± SE, n=9 mice/group). The mice treated with the fusion protein showed a 121% increase in placing score, relative to the saline treated mice, on the lesioned side (Figure 3).

The striatal TH enzyme activity in the left, or non-lesioned striatum, 5289 ± 422 pmol/hr/mg protein, in the saline treated mice was not significantly different from the TH enzyme activity in the left striatum for the fusion protein treated mice, 5919 ± 275 pmol/hr/mg protein (mean ± SE, n=9 mice/group) (Figure 4). Similarly, there was no difference in the TH enzyme activity in the frontal cortex for the saline treated mice, 203 ± 13 pmol/hr/mg protein, vs the fusion protein treated mice, 209 ± 11 pmol/hr/mg protein (Figure 4). However, the fusion protein treatment caused a 272% increase in striatal TH enzyme activity in the striatum on the lesioned side, from 1135 ± 192 pmol/hr/mg protein, in the saline treated mice, to 4221 ± 725 pmol/hr/mg protein, in the fusion protein treated mice (Figure 4).

3. Discussion

The results of this investigation demonstrate the known neuroprotective properties of intracerebrally injected GDNF in experimental PD (Hoffer et al, 1994; Lapchak et al, 1997) can be replicated with intravenous administration of the neurotrophin, providing the GDNF is fused to a BBB molecular Trojan horse. GDNF alone does not cross the BBB, which has been shown in both mice (Kastin et al, 2003) and Rhesus monkeys (Boado and Pardridge, 2009). Owing to the BBB problem, the systemic administration of GDNF alone is not therapeutic in experimental PD (Dietz et al, 2006). Therefore, if GDNF is to exert therapeutic effects in brain following systemic administration, the neurotrophin must be re-engineered to cross the BBB. This is possible by fusion of the GDNF to a BBB MTH. The MTH used in the present study is a chimeric MAb against the mouse TfR, which is designated the cTfRMAb. The constant regions of the cTfRMAb are derived from mouse IgG1/kappa, and the variable regions of the cTfRMAb are derived from the rat 8D3 MAb against the mouse TfR (Boado et al, 2009). Prior work has shown that the penetration of the BBB by the 8D3 MAb is receptor-mediated and saturable (Lee et al, 2000).

The properties of a pharmacologically active MTH fusion protein have been recently reviewed (Pardridge, 2010). First, the affinity of the GDNF for the GFRα1 must be retained following fusion to the MTH. Fusion of GDNF to either the HIRMAb or the cTfRMAb has no effect on the affinity of GDNF for the GFRα1, and has no effect on the biological activity of GDNF in a bio-assay using a human neural cell line (Boado et al, 2008; Zhou et al, 2010). Second, fusion of the GDNF to the MTH must not impair binding of the MTH to the endogenous BBB receptor that initiates transport across the BBB. Fusion of GDNF to the cTfRMAb has no effect on fusion protein binding to the mouse TfR (Zhou et al, 2010). The cTfRMAb-GDNF fusion protein binds both the GFRα1 and the mouse TfR with low nM binding constants. Third, the brain uptake of the MTH must be sufficiently high so as to allow for pharmacological effects in brain at low systemic doses of the fusion protein. In the case of the cTfRMAb-GDNF fusion protein, the brain uptake, 3.1% ID/g, is high, owing to the high affinity of the cTfRMAb part of the fusion protein for the BBB TfR (Zhou et al, 2010). Fourth, the BBB receptor transport system that is targeted by the MTH must be a

transcytosis system, not an endocytosis system. Potential MTHs that are actively endocytosed by cultured cells in vitro may not be transported across the BBB in vivo. The cationic tat import peptide is actively taken up by cultured cells but is not transported across the BBB in vivo (Lee and Pardridge, 2001). The poor in vivo transport properties of the tat peptide may explain the failure to observe neuroprotection in a mouse model of PD following systemic administration of a fusion protein of GDNF and the tat peptide (Dietz et al, 2006).

The use of a receptor-specific MAb as the BBB Trojan horse has certain advantages over non-IgG Trojan horses. Fusion of the amino terminus of the mature GDNF to the carboxyl terminus of the heavy chain of the targeting IgG places the GDNF in a dimeric configuration (Boado and Pardridge, 2008), which replicates the GDNF dimer that activates the GFRα1 (Eketjall et al, 1999). The fusion of GDNF to an IgG such as the HIRMAb, for humans, or the cTfRMAb, for mice, takes advantage of the presence of amino acid sequences, called Tregitopes, within the constant region of the IgG heavy chain that induce immune tolerance (DeGroot et al, 2008). The HIRMAb-GDNF fusion protein has been administered to Rhesus monkeys at high doses of 50 mg/kg IV, and no toxicity or any effect on glycemic control was observed (Pardridge and Boado, 2009).

The efficiency of the trans-vascular intravenous (IV) delivery of GDNF to brain in PD is illustrated by comparison of the dose of GDNF administered IV in this study with the dose of GDNF administered by trans-cranial intra-cerebral injection in experimental PD. The IV injection dose of the cTfRMAb-GDNF fusion protein administered in this study is 30 ug/mouse (Methods). Since the GDNF moiety comprises 17% of the cTfRMAb-GDNF fusion protein (Zhou et al, 2010), the dose of GDNF in this study is 5 ug/mouse IV. In contrast, the intra-cerebral injection dose of GDNF is 10 ug/brain in a mouse model of PD (Date et al, 1998), and is 100–1000 ug/brain in a rat model of PD (Hoffer et al, 1994; Lapchak et al, 1997). The low efficiency of trans-cranial drug delivery is attributed to the diffusion limitations within brain (Salvatore, et al, 2006). Conversely, owing to the short distances, e.g. 40 microns, between capillaries within the brain, diffusion plays no role in drug distribution in brain following trans-vascular delivery (Pardridge, 2002).

GDNF is delivered to all regions of brain following trans-vascular drug delivery to the brain. However, GDNF delivery to off-target regions of brain will generate biological effects only if the GDNF receptor, GFRα1, and its associated tyrosine kinase, c-ret, are expressed in the off-target region. While the GFRα1 is widely up-regulated following stroke or brain trauma, there is minimal expression of GFRα1 in normal brain (Arvidsson et al, 2001; Cheng et al, 2008). The GDNF target kinase, c-ret, is not detectable in control human brain, although its expression in control rat brain is measureable (Yang et al, 2006). In the present study, the trophic effects of trans-vascular GDNF are limited to the lesioned striatum, and there is no change in TH enzyme activity in either the frontal cortex or the non-lesioned striatum (Figure 4). These results show that trans-vascular GDNF delivery to brain does not result in off-target effects on TH expression.

In summary, the cTfRMAb-GDNF fusion protein is brain-penetrating form of GDNF that is specifically active in the mouse. A prior study showed a high brain uptake of the fusion protein of 3.1% ID/gram (Zhou et al, 2010). The present study demonstrates that chronic intravenous administration of the cTfRMAb-GDNF fusion protein in a mouse model of experimental PD is therapeutic.

4. Experimental procedures

4.1 6-hydroxydopamine model and treatment

All procedures were approved by the UCLA Animal Research Committee. Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) weighing 26–34 g were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Animals received a unilateral intra-cerebral injection of a total of 12 μg of 6-hydroxydopamine·HBr (Sigma Chemical Co.) dissolved in 0.02% ascorbic acid in 0.9% saline. The 6-hydroxydopamine (6 μg in 2 μL) was injected into the right striatum at 2 locations as described previously (Lundblad et al, 2004). The toxin was injected into the striatum at sites with the following stereotaxic coordinates: +1.0 mm relative to bregma, 2.1 mm relative to midline, 2.9 mm below the skull surface (site 1); +0.3 mm relative to bregma, 2.3 mm relative to midline, 2.9 mm below the skull surface (site 2). Mice were treated intravenously with either saline (n=9 mice), or the cTfRMAb-GDNF fusion protein, 1.0 mg/kg (n=9 mice), every 2 days over the following 3 weeks, with the first dose given 1 hour after toxin injection into the brain. Drug was injected IV via the tail vein in a volume of 50 uL/mouse. The mice were euthanized at 3 weeks following toxin administration for measurement of striatal TH enzyme activity.

The genetic engineering of the cTfRMAb-GDNF fusion protein has been described previously (Zhou et al, 2010). The 134 amino acid mature human GDNF was fused to the carboxyl terminus of the heavy chain of the IgG, as described by Boado et al (2008). There is a 93% amino acid identity between murine mature GDNF (Genbank NP_034405) and human mature GDNF (Genbank P39905), and a 93% amino acid identity between the murine GFRα1 extracellular domain (Genbank P97785) and the human GFRα1 extracellular domain (Genbank NP_665736). The cTfRMAb-GDNF fusion protein was expressed by stably transfected CHO cells cultured in serum free medium, and was purified by protein G affinity chromatography, as described previously (Zhou et al, 2010). The residual CHO host protein content in the purified fusion protein was measured by ELISA (Cygnus Technologies, Southport, NC), and was 24 ng per mg fusion protein, which is also expressed as parts per million (PPM). The DNA content in the fusion protein was measured by real time PCR using CHO cell genomic DNA as the assay standard, and was <0.05 PPM. The fusion protein was formulated in 0.01 M Tris buffered saline, pH=5.5, and was stored either sterile filtered at 4ºC or at −70ºC.

4.2 Behavioral testing

Beginning 1 week after the toxin administration, mice were tested weekly for apomorphine- and amphetamine-induced rotation, which was performed on separate days. For the apomorphine testing, mice were administered apomorphine (0.6 mg/kg) injected subcutaneously. Full (360°), contralateral rotations only were counted over 20 min, starting 5 min after apomorphine administration. For the amphetamine testing, mice were administered amphetamine (2.5 mg/kg) injected intraperitoneally. Full (360°), ipsilateral rotations only were counted over 20 min, starting 5 min after amphetamine administration. Mice were individually identified so that the rotations per minute (RPM) before and after fusion protein therapy could be compared for each mouse.

A vibrissae-elicited forelimb placing trial was carried out as described previously for rats (Anstrom et al, 2007). Each session included 120 trials (60 left side and 60 right side) in which a forelimb motor response to ipsilateral facial whisker stimulation was scored. In trials scored as a “3”, paw pads made full contact with table top. In trials scored as a “2”, paw pads do not make contact with the table. In trials scored as a “1” the limb moves forward only. In trials scored as a “0”, the limb does not move. A maximal possible score is 180, wherein the paw pad makes full contact with the table top in all 60 trials.

4.3 Tyrosine hydroxylase enzyme activity

TH enzyme activity in mouse brain striatum (left and right side) and in frontal cortex was measured with [3,5-^{3}H]-L-tyrosine (Perkin Elmer, Boston, MA) as substrate. The labeled [^{3}H]-water and L-DOPA was separated with a charcoal separation technique, as described previously (Zhang et al, 2003). Left and right striatum, and frontal cortex were removed and homogenized in 1.0 mL of 5 mM KH_2PO_4/pH=6.2/0.2% Triton X-100, followed by centrifugation. An aliquot of the supernatant was removed for protein content, which was measured with the bicinchoninic acid assay (Pierce Protein Research Products, Rockford, IL). Dithiothreitol was added to the supernatant to 0.1 mM, and the supernatant was stored at −20ºC. The final buffer composition of the TH enzyme activity assay included 0.4 mg/mL β-NADPH (Sigma N1630), 1 mM (6R)-5,6,7,8-tetrahydrobiopterin (Sigma T4425), 20,000 units/mL bovine liver catalase (Sigma C9322), 20 uM $Fe(NH_4)_2(SO4)_2$ (Sigma F1543), 10 uM L-tyrosine, and 13.3 uCi/mL of 3H-L-tyrosine in 0.03 M KH_2PO_4/pH=6.2. TH enzyme activity was measured at 37ºC for 30 min, and expressed as pmol/hour/mg protein.

4.4 Statistical analysis

Statistical differences between saline and fusion protein treated mice were determined with Student's t-test.

Acknowledgments

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Abbreviations

BBB	blood-brain barrier
GDNF	glial derived neurotrophic factor
PD	Parkinson's disease
MAb	monoclonal antibody
HIR	human insulin receptor
TfR	transferrin receptor
cTfRMAb	chimeric MAb against the mouse TfR
TH	tyrosine hydroxylase
GFR	GDNF receptor
CHO	Chinese hamster ovary
IV	intravenous
MTH	molecular Trojan horse
ID	injected dose
PPM	parts per million
RPM	revolutions per minute

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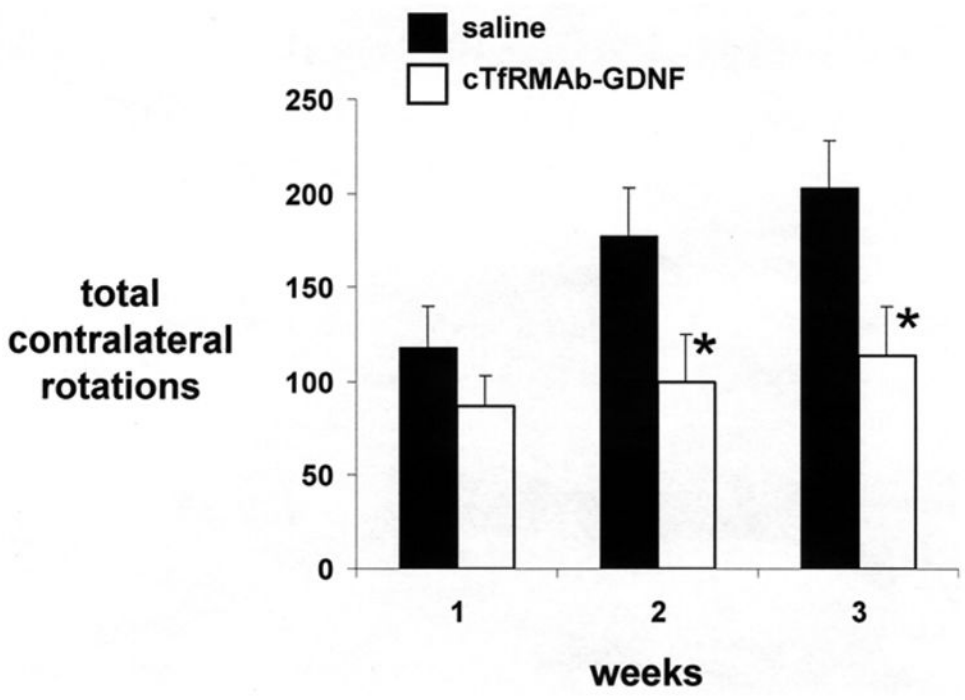

Figure 1.

Rotation measurements following the administration of apomorphine for PD mice treated with either saline or the cTfRMAb-GDNF fusion protein. Data are mean ± S.E. (n=9 mice/group). Statistical differences from the saline treated animals at 2 and 3 weeks are $p<0.05$ (*).

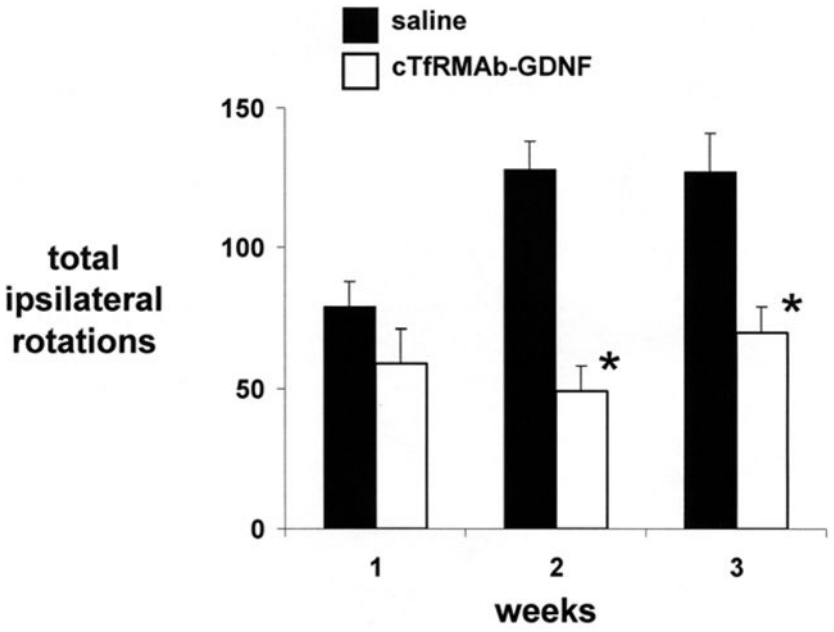

Figure 2.
Rotation measurements following the administration of amphetamine for PD mice treated with either saline or the cTfRMAb-GDNF fusion protein. Data are mean ± S.E. (n=9 mice/group). Statistical differences from the saline treated animals at 2 and 3 weeks are $p<0.005$ (*).

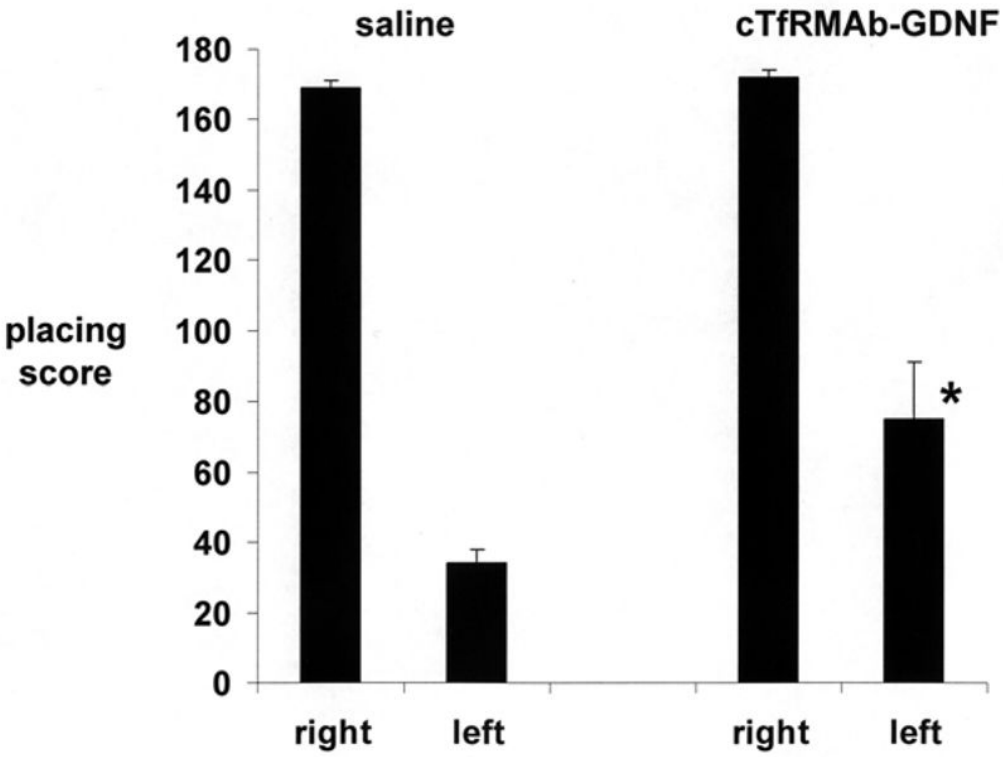

Figure 3.

Vibrissae-elicited forelimb placing test scores for right side, which is ipsilateral to the toxin lesion, and the left side, which is contralateral to the toxin lesion, for the saline and cTfRMAb-GDNF fusion protein treated mice. All scores were measured at 3 weeks following toxin injection. Data are mean ± S.E. (n=9 mice/group). Statistical differences from the saline treated animals are $p<0.05$ (*).

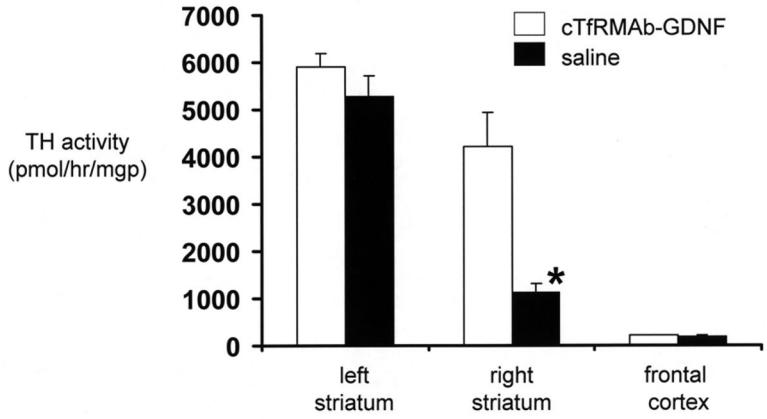

Figure 4.

Striatal TH enzyme activity on the lesioned side and the non-lesioned side in the striatum and in the frontal cortex in mice treated with either saline or the cTfRMAb-GDNF fusion protein. Brain TH activity was measured at 3 weeks after toxin administration. Data are mean ± S.E. (n=9 mice/group). Statistical differences from the saline treated animals in the right striatum are $p<0.001$(*).