Six-Month Continuous Intraputaminal Infusion Toxicity Study of Recombinant Methionyl Human Glial Cell Line-Derived Neurotrophic Factor (r-metHuGDNF) in Rhesus Monkeys


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ABSTRACT

Recombinant human glial cell line-derived neurotrophic factor (r-metHuGDNF) is a potent neuronal growth and survival factor that has been considered for clinical use in the treatment of Parkinson’s disease (PD). Here we present results of a 6-month toxicity study in rhesus monkeys conducted to support clinical evaluation of chronic intraputaminal infusion of r-metHuGDNF for PD. Monkeys (6–9/sex/group) were treated with 0 (vehicle), 15, 30, or 100 µg/day r-metHuGDNF by continuous unilateral intraputaminal infusion (150 µl/day flow rate) for 6 months; a subset of animals (2–3/sex/group) underwent a subsequent 3-month treatment-free recovery period. Notable observations included reduced food consumption and body weight at 100 µg/day and meningeal thickening underlying the medulla oblongata and/or overlying various spinal cord segments at 30 and 100 µg/day. In addition, multifocal cerebellar Parkinje cell loss (with associated atrophy of the molecular layer and, in some cases, granule cell loss) was observed in 4 monkeys in the 100-µg/day group. This cerebellar finding has not been observed in previous nonclinical studies evaluating r-metHuGDNF. The small number of affected animals precludes definitive conclusions regarding the pathogenesis of the cerebellar lesion, but the data support an association with r-metHuGDNF treatment.

Keywords. GDNF; Parkinson’s disease; toxicity; toxicity; cerebellum; Purkinje cell; monkey; infusion; putamen; rhesus.

INTRODUCTION

Glia1 cell line-derived neurotrophic factor (GDNF) is a potent endogenous neurotrophic factor that was first identified based on its ability to enhance the survival of dopaminergic neurons in dissociated rat embryonic midbrain cultures (Lin et al., 1993). Because of this characteristic, the therapeutic potential of exogenously administered GDNF in neurodegenerative disorders has been examined, particularly in the con-

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GDNF was generally well tolerated, although transient body weight loss was noted in drug-treated animals (Gash et al., 1996; Zhang et al., 1997). However, symptoms began to return toward baseline by 3 weeks postdose. When GDNF was administered as repeated monthly ICV injections, dose-dependent functional improvements were maintained in MPTP-lesioned monkeys (Gash et al., 1996; Zhang et al., 1997), thus demonstrating that repeated ICV administration of GDNF can effectively reduce Parkinsonian symptoms in nonhuman primate PD models. Moreover, GDNF was generally well tolerated, although transient body weight loss was noted in drug-treated animals (Gash et al., 1996; Zhang et al., 1997).

Grondin et al. (2002) evaluated the effects of continuous infusion of GDNF into the lateral ventricle (unilateral) or the putamen (bilateral) of monkeys with stable MPTP-induced lesions. ICV and intraputaminal (IPu) infusion of r-metHuGDNF were both shown to promote
functional improvements in parkinsonian features, increase the size and number of nigral neurons, and increase dopamine (and its metabolite) levels and the density of tyrosine hydroxylase-positive fibers in the striatum (Grondin et al., 2002). Furthermore, continuous infusion (ICV or IPu) of r-metHuGDNF was well tolerated by lesioned animals (Grondin et al., 2002); the body weight loss evident after bolus injections (Gash et al., 1996) was not observed.

Based on these data, initial clinical studies were conducted to evaluate the therapeutic potential of r-metHuGDNF in PD when delivered by repeated bolus ICV administration (Brundin, 2002; Kordower et al., 2000; Nutt et al., 2003). These clinical studies were supported by a comprehensive panel of nonclinical ICV toxicity studies. Subsequent clinical studies evaluated continuous IPu infusion of r-metHuGDNF in subjects with PD (Gill et al. 2003; Patel et al. 2005; Lang et al., 2006). To support clinical development of r-metHuGDNF administered by IPu infusion, the original ICV nonclinical toxicity package was supplemented with a series of IPu infusion toxicity studies that culminated in a pivotal 6-month study in rhesus monkeys.

The spectrum of drug-related effects observed in the pivotal IPu toxicity study was largely equivalent to that seen in shorter-term IPu infusion studies and in the bolus ICV administration studies. However, cerebellar neuron loss was observed in animals treated with the highest evaluated dose (100 µg/day) of r-metHuGDNF. The present paper summarizes and discusses salient results of this study.

MATERIALS AND METHODS

This study was conducted as part of the nonclinical toxicology program supporting the clinical development and potential registration of r-metHuGDNF for chronic IPu infusion in patients with Parkinson’s disease. The toxicology program was designed in accordance with International Conference of Harmonization Guideline S6 (Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals). The study was conducted in compliance with global Good Laboratory Practice (GLP) regulations, including those defined by the United States Food and Drug Administration (21 CFR Part 58).

Animals

All procedures involving animals were consistent with the Guide for the Care and Use of Laboratory Animals, DHHS, (NIH) No. 86-23 and the Animal Welfare Act (9 CFR 3) and were approved by the Animal Care and Use Committee of Northern Biomedical Research, Inc. (Muskegon, MI), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Seventy-two (36 male and 36 female) rhesus monkeys (Macaca mulatta) were acquired from Covance Research Products (Alice, TX), quarantined for at least 1 month before study initiation, and evaluated by standard methods for tuberculosis, herpes B virus infection, and parasitism. The monkeys were approximately 3 to 7 years old and weighed 3.37 to 7.65 kg at the time of study initiation (prior to surgical implantation of the drug delivery system). Animals were fed biscuits of PMI Certified Primate Diet 5048 (Richmond, IN) and supplemented weekly with vitamin C (CVS Pharmacy, Woonsocket, RI). In addition, fruits or vegetables were offered to entice food consumption if treatment-related weight loss necessitated supplementation. Filtered tap water was provided ad libitum. Environmental conditions in the animal room (temperature, humidity, and air changes) were controlled and monitored and did not depart significantly from ranges recommended in the Guide for the Care and Use of Laboratory Animals, DHHS, (NIH) No. 86-23. Lighting was automatically controlled to provide 12 hours of light and 12 hours of darkness.

General Study Design and Dosing Paradigm

To support chronic clinical use (per relevant ICH guidelines), the treatment period selected for the study was 26 weeks (~6 months). Nine animals per sex were initially assigned to 1 of 4 treatment groups by standard body weight randomization procedures (based on presurgical body weights). Subsequently, 3 animals/sex in each group were randomized into the recovery portion of the study using computer-generated random numbers. Animals were treated with vehicle or 1 of 3 doses of r-metHuGDNF by continuous unilateral IPu infusion (details regarding the infusion system appear below) for 26 weeks. Surviving main study animals in each group were necropsied at the end of the treatment period. Surviving recovery animals in each group underwent a subsequent 13-week (~3-month) treatment-free period, during which infusion pumps were emptied and flow was discontinued, to assess the reversibility or delayed occurrence of drug or infusion-related findings.

Doses of 0 (vehicle, citrate-buffered saline [CBS]), 15, 30, or 100 µg/day (concentrations of 0, 100, 200, and 667 µg/ml, respectively) of r-metHuGDNF were administered to the left putamen by continuous infusion at a rate of 6.25 µl/hr (150 µl/day). Lyophilized r-metHuGDNF (Amgen Inc., Thousand Oaks, CA) was diluted in 10 mM CBS to produce the required infusion solutions and stored at 4°C prior to use. Dosing solutions (vehicle and drug) were prepared, and infusion pumps were emptied and refilled, every 2 weeks.

Infusion rate and dose selection for this study were based on a previous 1-month IPu infusion toxicity study in rhesus monkeys in which 500 µg/day was associated with a single mortality and excessive body weight loss, and 100 µg/day was generally well tolerated. Therefore, 100 µg/day was selected as the high dose for the 6-month study. The lower doses of 15 and 30 µg/day were selected to evaluate potential dose-response relationships. The flow rate selected for the 6-month study was approximately one-half of the infusion rate used in the 1-month IPu infusion toxicity study (in which no flow rate-related problems were noted).

Estimated parameters on the study included clinical observations (daily), body weight (weekly), food consumption (daily), physical examinations (prestudy, postsurgery, Weeks 1, 4, 13, 26, 33, and 39), neurological examinations (parameters included level of consciousness, eye tracking, motor function, pupillary reflex, orbicularis oculi reflex, corneal reflex, sensory foot reflex, knee jerk, cutaneous reflex, proprioceptive reflex, and tail reflex; presurgery, postsurgery, Weeks 1, 4, 13, 26, 33, and 39), MRI scans (prestudy, postsurgery, Weeks 4, 13, 26, 33, and 39), ophthalmology (prestudy, Week 26), electrocardiograms (prestudy, Weeks 13, 26, and 39), clinical pathology (serum chemistry, hematology, coagulation, urinalysis; postsurgery, Weeks 4, 13, 26, 33, and 39), cerebrospinal fluid (CSF) analyses (cell count, chemistry, and protein; predose, Weeks 26, and 39), plasma r-metHuGDNF concentrations (Days 1, 2, 4, 8, and 15, 33, and 39), cerebrospinal fluid (CSF) analyses (cell count, chemistry, and protein; predose, Weeks 26, and 39), plasma r-metHuGDNF concentrations (Days 1, 2, 4, 8, and 15,
Delivery Device Implantation

Stereotaxic surgical procedures were used to implant an intraparenchymal catheter unilaterally into the left putamen of each monkey on the study. At catheter implantation, the animals were pretreated with atropine sulfate (sc, 0.04 mg/kg; Vedo, Inc., St. Joseph, MO), and approximately 15 minutes later, ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) was administered (im, 8.0 mg/kg) to induce sedation. The animals were administered 1.5% to 2.0% halothane (Halocarbon Laboratories, River Edge, NJ) or isoflurane (Vedo, Inc., St. Joseph, MO) in oxygen (delivered at 1 L/min) to achieve and maintain a surgical plane of anesthesia. Prednisolone sodium succinate (iv, 30 mg/kg; Pharmacia & Upjohn Company, Kalamazoo, MI) and flunixin meglumine (im, 2 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) were administered before surgery. A dorsal, sagittal incision was made over the calvaria and a 1.0-mm outer diameter, 0.6-mm inner diameter polyurethane intraparenchymal catheter (Model 8910A; Medtronic Neurological, Minneapolis, MN) was inserted via a 4 to 5 mm diameter craniotomy. The catheter was advanced posterior/inferior obliquely to the putamen in the left hemisphere and anchored in the craniotomy and to the skull with dental acrylic (Dental Manufacturing Co., Worth, IL).

The intraparenchymal catheter was joined with a stainless steel connector to the pump catheter (Medtronic Neurological, Minneapolis, MN); the connection was secured with a steel suture. The pump catheter traversed posterior in the subcutaneous tissue and terminated in a refillable Synchromed II pump (Model 8637-20; Medtronic Neurological, Minneapolis, MN) implanted in the left lateral paralumbar fossa between the internal and external abdominal oblique muscles.

The skin was closed with sutures and cyanoacrylic tissue adhesive (Vetbond Tissue Adhesive, 3M, St. Paul, MN). Upon recovery from anesthesia, the animals were given butorphanol tartrate (im, 0.05 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) for analgesia and a postsurgical antibiotic, cefotiofur sodium (im, 5.0 mg/kg BID; Pharmacia & Upjohn Co., Kalamazoo, MI).

Due to the large number of animals on the study, surgical implantation of the drug-delivery systems (requiring approximately 2 hours per animal) was conducted in three 4-day blocks (block 1: animals of both sexes designated for recovery groups; block 2: males in non-recovery groups; block 3: females in non-recovery groups). Within each of the 3 blocks, implantations were conducted beginning with the high-dose group, and progressing sequentially through the lower dose groups to the vehicle-treated control group to minimize the potential for contamination of the vehicle-treated group and accommodate logistical requirements for dosing preparation and pump filling. Treatment with the test article (or vehicle) was initiated approximately 4 weeks postsurgery in all animals.

Magnetic Resonance Imaging (MRI) Scans

Baseline MRI data (collected 2 to 4 weeks before implantation surgeries) were used to target the putamen and to screen the study population for pre-existing abnormalities. Posturgical MRI data collected during the treatment and recovery periods were used to confirm catheter placement and system patency, to monitor fluid flow at the site of infusion, and to noninvasively monitor effects of infusion. MRI scans were performed using a mobile MRI unit (Picker, 1.0T; Philips Medical Systems, Cleveland, OH). The following scans were completed for baseline, treatment period, and recovery period evaluations: T-1 weighted coronal, Fast Spin Echo (FSE) T-2 weighted coronal, and T-1 weighted coronal with gadolinium (gadodiamide 287 mg/ml; Amersham Health AS, Oslo, Norway; 0.1 mMol/kg, 0.2 ml/kg). In addition, T1-weighted sagittal scans were conducted at baseline to facilitate target mapping. All MRI slices were completed at 3.0 mm thickness with a 0.5 mm gap.

Each fasted animal was anesthetized with medetomidine hydrochloride (im, 0.08 mg/kg; Pfizer Animal Health, Exton, PA) and ketamine hydrochloride (im, 2.5 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and then intubated. A heparinized intravenous catheter (infused with physiologic saline) was placed in the saphenous vein. For baseline scans, the animals were placed in an MRI-compatible stereotaxic frame. Gadolinium contrast was injected through the saphenous catheter immediately prior to contrast MRI scans. After the scans, the animals were extubated, and the catheters were removed. Anesthesia was reversed with atipamezole hydrochloride (im, 0.2 mg/kg; Pfizer Animal Health, Exton, PA).

Plasma and CSF Analysis for r-metHuGDNF Concentrations

R-metHuGDNF concentrations in the systemic circulation and CSF were determined at multiple timepoints. Blood samples were collected from a peripheral vein (into tubes containing potassium-EDTA as an anti-coagulant) and processed to plasma for analysis. CSF concentrations of r-metHuGDNF were determined in samples collected via cisterna magna taps (approximately 3 ml was collected; 0.5 ml was used for r-metHuGDNF concentration analyses, and the remaining volume was used for antibody, cell count, and chemistry analyses). Taps were performed using a 20-gauge spinal needle while the animals were under injectable (medetomidine hydrochloride/ketamine hydrochloride) or inhaled (1.5% to 2.0% halothane or isoflurane in oxygen delivered at 1 L/min) anesthesia.

The assay for r-metHuGDNF in plasma and CSF was a conventional solid phase sandwich enzyme immunoassay (Swanson et al., 2002). Microwell plates were coated with a monoclonal (murine) anti-r-metHuGDNF antibody (MAB 212, R&D Systems, Minneapolis, MN) as the capture antibody. The r-metHuGDNF in the samples was captured by the immobilized monoclonal antibody. After unbound r-metHuGDNF was removed by washing the wells, a biotinylated anti-r-metHuGDNF polyclonal (goat) antibody (BAF 212, R&D Systems, Minneapolis, MN) was added to the
wells for detection of the captured r-metHuGDNF. This was followed by addition of streptavidin-conjugated horseradish peroxidase to bind on the complex. The substrate solution reacts with the peroxidase to create a colorimetric signal that is proportional to the amount of r-metHuGDNF bound by the capture antibody in the initial step. The lower limit of quantification (LLOQ) of the assay was between 100 and 320 pg/ml.

**Serum and CSF Analysis for GDNF Antibodies**

Anti-r-metHuGDNF antibodies were evaluated in the systemic circulation and CSF at multiple time points. Blood samples were collected from a peripheral vein (approximately 2 ml with no anticoagulant) and processed to serum for analysis. CSF samples were collected as described above via cisterna magna taps (approximately 3 ml collected; 1 ml used for antibody analyses, remaining volume used for r-metHuGDNF concentration, cell count, and chemistry analyses). Two validated assays were used to test for antibodies to r-metHuGDNF. The first assay was an immunoblot assay to detect the presence of binding antibodies. The second assay was a cell-based bioassay to detect potential neutralizing activity (in vitro) of detected antibodies.

The binding immunoassay was run on a Biacore 3000 (Biacore International, AB, Uppsala, Sweden) biosensor-based instrument that uses surface plasmon resonance (SPR) as the detection principle. With this detection principle, r-metHuGDNF was covalently attached to a carboxymethyl-dextran-coated sensor chip. Changes in refractive index due to mass accumulation at the sensor surface occurred upon anti-r-metHuGDNF antibody binding; this binding interaction was monitored and recorded on a sensorgram.

The approximate sensitivity of this immunoassay was 0.78 μg/ml of rabbit positive control antibody in neat negative control serum and 0.25 μg/ml in neat CSF. The neutralizing antibody bioassay used 32D REG c11 cells, a clone of murine 32Dc13 cells transfected with the HuGDNF receptor alpha unit as well as a chimeric co-receptor consisting of an extracellular domain of proto-oncogene Ret and the transmembrane domain and truncated intracellular signaling domain of the EPO receptor. Cells responded to the stimulation r-metHuGDNF and/or murine interleukin-3 (mIL-3) with proliferation. The proliferation was measured as adenosine triphosphate levels in cell lysate using the ViaLight Plus luminescence assay (Cambrex Bioscience, East Rutherford, NJ) and analyzed on a TopCount luminometer (Perkin Elmer, Wellesley, MA).

In order to assess neutralizing activity, serum or CSF samples were diluted and incubated with known concentrations of r-metHuGDNF and mIL-3 followed by addition to the 32D REG c11 cells. If a sample inhibited r-metHuGDNF-induced proliferation, but showed no effect on mIL-3-induced proliferation, the sample was reported as positive for the presence of anti-r-metHuGDNF neutralizing activity. If a sample showed inhibition of both r-metHuGDNF and mIL-3 induced proliferation, the sample was considered to contain non-specific inhibitory factors and was considered to be negative for anti-r-metHuGDNF neutralizing antibodies. The approximate sensitivity of this bioassay was 0.8 μg/ml of rabbit positive control antibody in neat negative control serum and 0.4 μg/ml in neat CSF.

**Necropsy, Histopathology, and Immunohistochemistry**

All animals were subjected to a full necropsy. Scheduled necropsies occurred at the end of the treatment period and at the end of the recovery period. The study pathologist was in attendance for all scheduled necropsies. Animals were sedated with ketamine hydrochloride (im, 8.0 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), administered an intravenous bolus injection of heparin sodium at 200 IU/kg (Elkins-Sinn, Inc., Cherry Hill, NJ), and maintained on 1.5% to 2.0% halothane (Halocarbon Laboratories, River Edge, NJ) or isoflurane (Vedco, Inc., St Joseph, MO) in oxygen (delivered at 1 L/min). Whole-body perfusion fixation (via the left cardiac ventricle) was performed with 0.001% sodium nitrite in saline (200 ml/min for 2 min; VWR West Chester, PA) followed by 10% neutral-buffered formalin (200 ml/min for 10 min; VWR, West Chester, PA). Animals were subsequently infused (200 mL/min for 2 min) with a 10% sucrose (Sigma Chemical Company, St Louis, MO) solution in 80:20 NaCl (0.9%):PBS (pH 7.2).

Adrenal glands, brain, heart, kidneys, liver, ovaries, pituitary gland, spleen, testes, thymus, and thyroid gland (with parathyroid) were weighed from each animal. Organ-to-body weight and organ-to-brain weight ratios were calculated for animals subjected to a scheduled necropsy.

Approximately 45 organs and tissues (or samples thereof, as appropriate) from each animal were collected for subsequent microscopic pathology evaluation. Organs and tissues from outside the CNS were preserved in 10% neutral-buffered formalin. Brains and spinal cords were preserved in a 10% sucrose solution to facilitate immunohistochemical analyses.

Tissues collected from outside the CNS were trimmed, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for standard microscopic pathology evaluation. Eight full coronal (transverse) brain slices (~3 to 4 mm/slice) were taken from each animal and embedded in paraffin blocks. Five slices spanning from forebrain to midbrain (including samples of caudate/putamen), 1 slice represented midbrain (at the level of the substantia nigra), and 2 slices spanned the hindbrain (typically including pons, cerebellum, and medulla oblongata).

Single sections (5 μm) from each of the blocks were mounted on slides and stained with H&E for routine morphological evaluation, Luxol fast blue-periodic acid Schiff (LFB-PAS) for myelin, Fluoro-Jade B for staining degenerating and/or necrotic neurons and neuronal cell processes, anti-glial fibrillary acidic protein (GFAP) for detection of astrocytes for analysis of gliosis and the extent of injury to the CNS, and anti-GDNF for detection of administered r-metHuGDNF. Immunohistochemical staining for GFAP and GDNF was performed using commercially available polyclonal antisera (rabbit anti-human GFAP [Dako, Carpinteria, CA] and goat anti-human GDNF [R&D Systems, Inc., Minneapolis, MN]) and standard indirect immunohistochemical methods; slides were counterstained with hematoxylin.

Three levels of the spinal cord (cervical, thoracic, and lumbar), with transverse and oblique sections taken at each level, were embedded in paraffin, and serial sections (5 μm) were stained with H&E, LFB-PAS, anti-GFAP, Fluoro-Jade B, and anti-GDNF. Dorsal spinal nerve roots and ganglia (taken at mid-cervical, mid-thoracic, and mid-lumbar), tibial nerve,
sural nerve, sciatic nerve, and trigeminal ganglia from all animals were embedded in glycol methacrylate (GMA) blocks; sections (5 μm) were stained with H&E for evaluation. Remaining cerebellar tissue from all animals was trimmed and embedded in paraffin blocks as described for additional evaluation. Sections (5 μm) were taken from these blocks (2 to 3 per animal) and stained with H&E and anti-GFAP.

A rough estimate of the extent of cerebellar lesions in affected animals was generated using a Bioquant system (BIOQUANT Image Analysis Corporation, Nashville, TN). Briefly, cerebellar cortex area was outlined in digitized photomicrographs of H&E-stained slides (4 to 5 total slides available from each affected animal) on the Bioquant workscreen. Total cortical area was determined by applying a threshold that quantified the number of tissue-stained pixels within the outlined area. Application of the threshold effectively excluded unstaed area from calculations. The area of affected cortex was determined by the same general procedure. Areas from individual slides were summed to yield overall estimates of total and affected cortical areas. The area of affected cortex in each animal was calculated as the percent of the total cortical area measured on the available slides.

Statistical Methods

Body weights, body weight changes, food consumption, clinical pathology data, CSF cell count and chemistry, and absolute and relative organ weights were analyzed by a 1-way analysis of variance (ANOVA). If significant differences were apparent, each treated group was compared with the control group using Dunnett’s multiple comparison test. Analysis was 2-tailed, and statistical significance was defined as p ≤ 0.05.

RESULTS

In-Life Observations

At study initiation, 4 treatment groups were established with an equal ratio of males and females (6/sex/group in the main study and 3/sex/group in the recovery group); 3 animals were euthanized before initiation of dosing. In total, 13 animals (of 72 initially placed onto the study) died or were euthanized before the scheduled necropsies, leaving 59 for terminal analyses (Table 1). Twelve of these unscheduled deaths/euthanias were unrelated to drug treatment (pump-related complications). One animal in the mid-dose group was euthanized with clinical signs and clinical pathology findings consistent with disseminated intravascular coagulation (DIC). Microscopic evaluation of this animal revealed widespread hemorrhages. DIC has not been reported in previous studies with GDNF; therefore, it was believed to be due to a reaction to the presence of a human antigen (r-metHuGDNF) and not to the pharmacological activity of the molecule.

Clinical observations over the course of the study were unremarkable. Physical and neurological examinations revealed no remarkable findings. Electrocardiograms revealed no test article-related effects. All ophthalmologic examination findings were considered within normal limits. No toxicologically significant hematology, serum chemistry, coagulation, or urinalysis parameters were observed. Furthermore, CSF total cell count and chemistry profiles were unremarkable.

Dose-related body weight and food consumption reductions (data not shown) were observed in all drug-treated groups; however, toxicologically significant effects were limited to the high-dose group. Absolute mean body weights in all drug-treated groups were generally reduced, relative to controls, across most of the treatment period, although these reductions did not reach statistical significance (body weight ranges within groups were wide). The absolute body weight reductions in the high-dose group were appreciable (particularly in males); at the end of the treatment period, mean body weights in males and females were 16.4% and 11.7% lower, respectively, than in controls.

Statistically significant reductions in body weight change (relative to the control group) occurred in the high-dose group during the treatment period, with milder reductions (not statistically significant) evident in the low- and mid-dose groups. In fact, body weight loss (relative to predose) occurred during the first half of the treatment period in the high-dose group. Food consumption changes qualitatively paralleled the body weight effects, and the degree of body weight loss experienced in the high-dose group necessitated dietary supplementation for individual animals. With the exception of females in the control group, all groups gained weight during the recovery period.

MRI findings associated with infusion of vehicle and/or drug were noted at the infusion site in all groups. No remarkable findings were noted in areas of the brain distant from the infusion site. The typical finding was a diffuse spherical signal (on T-1 with gadolinium and T-2 FSE scans) approximately 3 mm in mean diameter and centered at the catheter tip. Specifically, on T-1 scans, an infusion-related increase in contrast uptake was noted in all groups (including controls), suggesting localized disruption of the blood brain barrier. On T-2 FSE scans, an infusion-related increased diffuse T-2 FSE hyperintense signal was noted in all groups. Interestingly, the hyperintense T-2 FSE signal was typically asymmetrical (Figure 1), with a bias towards the ipsilateral lateral ventricle. In both cases (T-1 and T-2 scans), the incidence and/or intensity of the signals were increased in the 100-μg/day dose group relative to the control, low-dose, and mid-dose groups. In addition, the MRI findings were not apparent at the end of the recovery period, suggesting that they were associated directly with the active infusion.

Plasma and CSF r-metHuGDNF Concentrations

Quantifiable concentrations of r-metHuGDNF were not detected in the plasma of animals treated with 0, 15, or 30 μg/day at any of the evaluated time points. Among the 18 animals in the high-dose group (including the 3 animals...
not surviving to scheduled necropsy), 9 had low, but detectable plasma concentrations at 1 or more of the 8 treatment period sampling time points (data not shown). No detectable concentrations of r-metHuGDNF were observed in plasma in any group at the end of the 3-month recovery period.

Mean concentrations of r-metHuGDNF in the CSF increased with increasing dose at Week 4 and at the end of treatment (Week 26) in the drug-treated groups (Table 2). The frequency of quantifiable concentrations also increased with increasing dose. Interestingly, mean levels of r-metHuGDNF in the CSF generally decreased from the 1- to 6-month
intervals (Table 2). Individual animal CSF concentrations were extremely variable, with considerable overlap across dose groups. Individual CSF concentrations in the high-dose and low/mid-dose groups are presented in Tables 6 and 7, respectively.

Serum and CSF Anti-r-metHuGDNF Antibodies

As expected of a recombinant human protein administered to rhesus monkeys, r-metHuGDNF was highly immunogenic, producing detectable binding and neutralizing antibodies in both the serum and CSF. The incidence of anti-r-metHuGDNF binding antibodies at 1 or more time points across the study was 88% (46/52) in the serum of drug-treated animals (Table 3). The incidence was dose-dependent, ranging from 69% at the low dose to 100% at the high dose. Approximately 40% (21/52) of these animals had neutralizing antibodies as determined in the bioassay.

Serum antibodies were often found on Day 2 of treatment (data not shown). Since antibodies do not usually develop so rapidly, it is likely that the animals were exposed to r-metHuGDNF during surgical implantation of the pump/catheter system or as a result of pump flushing procedures conducted during the approximate 4-week period between surgery and treatment initiation, which required the withdrawal and addition of dosing solutions through the skin and pump septum with a needle and syringe every 2 weeks.

The frequency of serum neutralizing antibody activity increased during the study to reach a maximum at the end of the treatment period (data not shown).

Approximately 14% (7/50) of drug-treated animals had anti-r-metHuGDNF binding antibodies in the CSF. All 7 animals that tested positive for binding antibodies in the CSF also tested positive in the neutralizing antibody assay, but the incidence did not appear to correlate with dose, since the 15- and 30-µg/day dose groups exhibited higher percentages of CSF binding and neutralizing antibodies than the 100-µg/day dose group. CSF samples taken at the recovery time point were negative for anti-r-metHuGDNF binding and neutralizing antibodies.

GROSS NECROPSY, IMMUNOHISTOCHEMISTRY, AND HISTOPATHOLOGY

Gross Necropsy and Organ Weights

Full necropsies performed on all animals at the end of treatment and end of recovery revealed no drug-related or otherwise notable gross findings. Likewise, organ weights were unremarkable.

GDNF Immunohistochemistry

Anti-GDNF immunohistochemistry staining in the brain was included in this study primarily to demonstrate intraparenchymal exposure to r-metHuGDNF, in the anticipated absence of meaningful systemic pharmacokinetic data. In addition, while not prospectively included in the study for this purpose, the results allowed a gross assessment of r-metHuGDNF distribution in the brain to facilitate identification of regions preferentially accumulating the molecule. A representative set of sections from an animal in the mid-dose group is shown in Figure 2.

Anti-GDNF staining was generally limited to the brain parenchyma at, and immediately surrounding, the infusion site in all drug-treated groups. The intensity and distribution of staining tended to increase with increasing doses; however, considerable individual animal variability was evident. Notably, no other regions of the brain showed appreciable staining. No GDNF immunoreactivity was detected in any tissue sections from vehicle-treated animals.

Where present, staining tended to be most pronounced in the white-matter tracts, suggesting that they offered less resistance to the movement of the infused study drug. In animals with pronounced inflammation at the catheter site, GDNF tended to be trapped within the zone of the inflammatory response. Staining was consistently present in the r-metHuGDNF-treated animals at the recovery necropsy, but was less pronounced than in the same dose groups at the end-of-treatment necropsy (data not shown).

Histopathology

Histopathologic evaluation was performed on all collected tissues from all animals, including the brain, spinal cord, and closely associated structures, and a full panel of tissues from outside the nervous system. Drug-related changes were limited to CNS tissues and meninges and immediately adjacent tissues of the peripheral nervous system (dorsal root entry zones).
FIGURE 2.—Anti-GDNF immunohistochemistry staining in a mid-dose (30 µg/day) male (1X objective). Note the limited intensity of staining in brain regions distant from the area immediately surrounding the infusion site. Coronal sections are labeled from A to H, progressing from rostral to caudal. The staining pattern shown is qualitatively representative of other drug-treated animals. The infusion site appears on the right side of the images as they are shown from a frontal orientation.
Drug-related microscopic findings in the CNS included an increased inflammatory reaction to the drug formulation (Figure 3), which would be expected with administration of a foreign protein into the CNS, and effects potentially attributable to the pharmacological activity of r-metHuGDNF that included thickened meninges (Figure 4) and loss of neurons in the cerebellar cortex (Figures 5, 6, and 7). The details of the notable effects appear next.

Other findings were noted in the brain; however, their appearance did not differ between control and drug-treated groups and were likely related to the drug-delivery system. These findings were primarily limited to the catheter tract or immediately adjacent areas, and will not be detailed. These observations included hemorrhage, gliosis/astrocytosis, and multinucleated giant cell infiltrates. Extracellular fluid adjacent to the catheter tract was also a consistent finding in all groups (including controls). While its observation could be associated with edema, the fluid buildup was more likely an accumulation of the infusate.

**Inflammation**

Minimal-to-mild inflammation was noted along the catheter tract and at the infusion site at the end of the treatment period in all groups, with an increase in overall incidence and/or average severity apparent in drug-treated groups (Figure 3). The inflammatory reaction was generally characterized by 1 or more of the following: increased (relative to controls) infiltrates of lymphocytes at the catheter tract, increased infiltrates of eosinophils at the catheter tract, increased perivascular infiltrates of lymphocytes adjacent to the catheter tract, and/or increased perivascular infiltrates of eosinophils adjacent to the catheter tract. Notably, the lymphocyte/eosinophil infiltrate differed only slightly, if at all, between the low-dose and control groups. Other components of the inflammatory reaction, including fibrosis, hemorrhage, macrophage and neutrophil infiltrates, gliosis/astrocytosis, and foreign body reaction (presence of multinucleated giant cells), were essentially similar between control and r-metHuGDNF-treated groups, or were variable and lacked a clear dose response.

At the recovery necropsy, the inflammatory response was largely comparable across all groups, including the control group, suggesting some degree of resolution during the untreated period (Figure 3c).

**Meningeal Thickening**

Minimal to mild meningeal thickening was observed along the anterior/ventral surfaces of the medulla oblongata and/or overlying various spinal cord segments in the mid- (30 µg/day) and high-dose (100 µg/day) groups (Figure 4). The thickening was characterized by increased cellularity of the meninges and was composed of polygonal and spindle-shaped cells, which tended to form clusters that appeared to be provisional neural structures. In the spinal cord, the findings were primarily noted in the vicinity of the dorsal root entry zones and were most consistently present in sections from the cervical and lumbar regions. The findings were noted at the end of treatment in 1 of 9 animals (1 of 4 males, 0 of 5 females) in the 30-µg/day dose group and in 6 of 10 animals (2 of 4 males, 4 of 6 females) in the 100-µg/day dose group. They were still present at the end of the recovery period in 3 of 5 animals in the 100-µg/day group (2 of 2 males, 1 of 3 females).
FIGURE 4.—Meningeal thickening and hypercellularity in mid-dose (30 µg/day) and high-dose (100 µg/day) groups (10× objective). Animals necropsied at the end of treatment (A to D) and recovery (E, F) were stained with H&E. (A) Control group male, lumbar spinal cord, dorsal surface showing normal meninges. (B) Mid-dose (30 µg/day) group male, cervical spinal cord, dorsal surface with arrow located at a focal area of hypercellularity due to spindloid hyperplasia. (C) High-dose (100 µg/day) group male, lumbar spinal cord, dorsal surface with arrows at areas of slight hypercellularity and an increase in thickness of the meningeal membranes. (D) High-dose (100 µg/day) group male, thoracic spinal cord, dorsal surface with arrows at areas of slight hypercellularity and an increase in thickness of the meningeal membranes. In (E) and (F), parenchyma sections from the meningeal surface of the medulla oblongata in control and high-dose (100 µg/day) group males necropsied at the end of the recovery period are shown, respectively. Increased cellularity at the meningeal surface due to spindloid cell hyperplasia was observed in the high-dose (100 µg/day) group animal necropsied at the end of recovery (F). Hyperplastic spindloid cells in the meninges were confirmed to be Schwann cells by electron microscopy in previous studies (data not shown).

Cerebellar Lesions

Multifocal cerebellar Purkinje cell loss (Figure 5) was observed in 4 animals in the high-dose group (Table 4); the Purkinje cell loss was minimal to moderate in severity and was associated with minimal to moderate atrophy of the molecular layer and granule cell loss at some foci (Table 5). The lesion was present in 1 of 4 males necropsied at the end of the treatment period, 1 of 2 males necropsied at the end
FIGURE 5.—Purkinje cell loss in the cerebellum of animals in the high-dose (100 µg/day) group necropsied at the end of recovery (40× objective). Note the normal Purkinje cells (arrows), the densely populated granule cell layer (G) and the uniform molecular layer (M) in the control-group brain section (A). In a female in the high-dose (100 µg/day) group necropsied at the end of recovery (B), there was a hypocellular granule cell layer (G), an absence of Purkinje cells, and a vacuolated and loosely arranged molecular layer (M).

of the recovery period, and 2 of 3 females necropsied at the end of the recovery period. The lesions were not apparent in the control, low-dose, or mid-dose groups at either necropsy interval.

Minimal to moderate astrocytosis (Table 5) was also present in areas of Purkinje cell loss. The astrocytosis was characterized by increased GFAP staining across the molecular layer in the areas of Purkinje cell loss (Figure 6). In areas where Purkinje cells were present adjacent to areas where they were absent, a pronounced difference in anti-GFAP staining was apparent. In some areas where Purkinje cells were lost, increased cellularity due to astrocyte proliferation (Bergmann gliosis) was present in the Purkinje cell layer. The Purkinje cell loss was not associated with increased Fluoro-Jade B staining (data not shown).

TABLE 4.—Incidence of cerebellar lesion at scheduled necropsies

<table>
<thead>
<tr>
<th>Dose (µg/day)</th>
<th>End of Treatment Period Necropsy</th>
<th>End of Recovery Period Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>0</td>
<td>0/5</td>
<td>0/6</td>
</tr>
<tr>
<td>15</td>
<td>0/4</td>
<td>0/6</td>
</tr>
<tr>
<td>30</td>
<td>0/4</td>
<td>0/5</td>
</tr>
<tr>
<td>100</td>
<td>1/4</td>
<td>0/6</td>
</tr>
</tbody>
</table>

TABLE 5.—Cerebellar lesion character, severity grade, and involved area in 4 affected high-dose (100 µg/day) group animals

<table>
<thead>
<tr>
<th>Lesion Character</th>
<th>Male 028 End of Treatment Period Necropsy</th>
<th>Male 034 End of Recovery Period Necropsy</th>
<th>Female 071 End of Recovery Period Necropsy</th>
<th>Female 072 End of Recovery Period Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purkinje cell loss</td>
<td>Mild</td>
<td>Minimal</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>Molecular layer atrophy</td>
<td>Minimal</td>
<td>Mild</td>
<td>Moderate</td>
<td>Minimal</td>
</tr>
<tr>
<td>Granule cell loss</td>
<td>Mild</td>
<td>Minimal</td>
<td>Moderate</td>
<td>Minimal</td>
</tr>
<tr>
<td>Astrocytosis</td>
<td>Mild</td>
<td>Minimal</td>
<td>Moderate</td>
<td>Minimal</td>
</tr>
<tr>
<td>% Cerebellar Cortex Affected</td>
<td>3%</td>
<td>1%</td>
<td>21%</td>
<td>3%</td>
</tr>
</tbody>
</table>
The severity of the cerebellar lesion varied considerably by focus and by animal (Table 5). Animal No. 071 (1 of the 2 females in the end-of-recovery necropsy group) was the most severely affected. Within individual animals, there were areas where only Purkinje cells were absent, or where atrophy of the molecular layer was seen in association with the Purkinje cell loss, as would be expected with loss of Purkinje dendritic branches. Interestingly, there were also foci where granule cell loss was apparent in association with the Purkinje cell loss and molecular layer atrophy.

The lesion was distributed among multiple cerebellar folia and the vermis, with the overall affected area of the cerebellum varying considerably among the 4 affected animals (Table 5; % cerebellar cortical involvement estimated from multiple low power sections; represented in part by Figure 7). The most extensively affected area (21% of the cerebellar cortex) was noted in 1 of the females in the end-of-recovery necropsy group (No. 071). This animal also had the most severe lesion. The least extensive lesion (approximately 1% of the cortex), was apparent in the single male in
the end-of-recovery necropsy group (No. 034). A clear pattern in overall cerebellar lesion distribution was not evident from evaluated sections.

**DISCUSSION**

Six months of continuous unilateral IPu infusion of r-metHuGDNF produced a spectrum of drug-related effects in rhesus monkeys. The nature of these effects was largely consistent with the findings of previous studies conducted with the molecule (unpublished data; Lapchak et al., 1997a; Zhang et al., 1997) and included reductions in food consumption associated with body weight decrements (at the high dose of 100 µg/day), inflammatory changes in the brain parenchyma along the catheter tract and at the infusion site (≥15 µg/day, i.e., all doses), and meningeal thickening along dorsal aspects of the spinal cord and underlying the medulla oblongata (≥30 µg/day). The meningeal thickening was considered to be due primarily to focally extensive sprouting of axons (sympathetic/sensory) and/or myelination of unmyelinated fibers (Hoke et al., 2003) and associated Schwann cell hyperplasia. This conclusion was based on morphologic presentation and electron microscopy observations in a previous study evaluating ICV-administered r-metHuGDNF (unpublished). Notably, these meningeal observations have also been described with the central administration of nerve growth factor (Day-Lollini et al., 1997; Winkler et al., 1997; Pizzo and Thal, 2004).

An additional observation of neuron loss in the cerebellar cortex was noted in the high-dose group (100 µg/day) in the present study. This lesion was characterized by loss of Purkinje cells with atrophy of the overlying molecular layer and, in some cases, associated cell loss in the granule cell layer. At affected foci, the lesion appeared as either Purkinje cell loss alone, Purkinje cell loss with associated proliferation of Bergmann’s glia/astrocytosis and astrocytosis and vacuolation in the molecular layer, or a more extensive, nearly full-thickness loss of the entire cerebellar cortex (molecular, Purkinje cell, and granule cell layers). The finding was not associated with Fluoro-Jade-positive neurons (Schmued and Hopkins, 2000) or microgliosis (Koestner and Jones, 1997) at, or adjacent to, affected sites, suggesting that neuronal degeneration was not ongoing at the time of scheduled necropsy (end-of-treatment or end-of-recovery).

Cerebellar lesions have not been observed in previous non-human primate toxicology studies (unpublished) evaluating administration of r-metHuGDNF by sc (daily doses up to 1000 µg/kg for 1 month with a 2-week recovery period), intrathecal (monthly bolus administration of up to 10,000 µg/dose for 3 months), or ICV (monthly bolus administration of up to 3000 µg/dose for 6 months with a 3-month recovery period) routes. Furthermore, cerebellar lesions were not observed in a 1-month IPu infusion study (with no recovery assessment) in rhesus monkeys, despite the fact that the highest evaluated dose in that study (500 µg/day) was 5 times the dose associated with the findings in the present study. In addition, the cerebellar lesion has not been reported in association with r-metHuGDNF treatment in rodent or non-human primate studies described in the published literature (Gash et al., 1995, 1996; Bjorklund et al., 1997; Rosenblad et al., 2000; Grondin et al., 2002). In the 3-month repeated intrathecal bolus study mentioned above, axonal degeneration was observed in the peripheral tracts of the lower spinal cord in the area immediately surrounding the administration site (data not shown).

The cerebellar injury occurred only in animals treated with the highest evaluated dose of 100 µg/day (Table 4). This dose-related appearance suggests that the lesion was a drug-related phenomenon. The lesion was observed in the high-dose group at the end-of-treatment necropsies in 1 of 4 males and 0 of 6 females and at the end-of-recovery necropsies in 1 of 2 males and 2 of 3 females. Therefore, 3 of the 4 affected animals were necropsied at the end of the recovery period that followed the 6-month treatment period. While the number of affected animals is small, these data nevertheless suggest that cessation of treatment during the recovery period (i.e., withdrawal) could have contributed to development of the lesion.

The underlying mechanism for this effect is not clear. However, the fact that the lesion was seen in 1 animal (No. 028) at the end of the treatment period argues that a formal recovery period was not a prerequisite. Infusion pump refill records (pumps were refilled every 2 weeks), pump-recorded functional information (e.g., volume administered, flow rate), infusion site GDNF immunohistochemistry, and plasma r-metHuGDNF concentration data all suggested that the drug was delivered to the striatum, as intended, and provided no evidence of an unintended cessation of treatment in this animal. Therefore, it is possible that the lesion was not a withdrawal-mediated effect, and that the higher incidence of affected animals in the recovery group is an artifact. The injury could conceivably have occurred, or at least been initiated, during the treatment period.

Comprehensive evaluations revealed no definitive associations between the many parameters on the study and the occurrence of the cerebellar lesion. These efforts, however, were complicated by the small number of affected animals. Two potentially noteworthy parameters are the antibody and r-metHuGDNF plasma and CSF concentration data. However, evaluation of individual antibody profiles in the serum and/or CSF (whether binding or binding and neutralizing) revealed no clear correlations with the presence or absence of the cerebellar lesion. Likewise, while systemic (plasma) concentrations of r-metHuGDNF were detectable only in the high-dose group, individual plasma levels did not correlate with the presence or absence of the cerebellar lesion.

Mean concentrations of r-metHuGDNF in the CSF of drug-treated groups increased with increasing dose (Table 2), suggesting that elevated CSF levels of r-metHuGDNF could be associated with the dose-related appearance of the cerebellar lesion. Elevated levels of r-metHuGDNF in the CSF secondary to IPu administration may have resulted from backflow along the catheter tract into the subarachnoid space due to resistance at the infusion site (Morrison et al., 1999) or from flow of interstitial fluid from the infusion site into the nearby lateral ventricle. Available data from the study do not allow definitive conclusions to be made in this regard. However, the T2-MRI hyperintense signal surrounding the infusion site showed a bias toward the lateral ventricle (Figure 1).

While group mean CSF concentrations of r-metHuGDNF increased with dose (Table 2), elevated individual CSF concentrations (Table 6) did not clearly correlate with the...
Table 6.—Individual concentrations (pg/ml) of r-metHuGDNF in cerebrospinal fluid of animals in the high-dose (100 µg/day) group

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>End of First Month of Treatment Period</th>
<th>End of 6-Month Treatment Period</th>
<th>End of 3-Month Recovery Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (028)</td>
<td>Affected Animals</td>
<td>2099</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Male (034)</td>
<td>End of Treatment</td>
<td>161775</td>
<td>BQL</td>
<td>BQL</td>
</tr>
<tr>
<td>Female (071)</td>
<td>End of Recovery</td>
<td>2940</td>
<td>4409</td>
<td>BQL</td>
</tr>
<tr>
<td>Female (072)</td>
<td>End of Treatment</td>
<td>1710</td>
<td>BQL</td>
<td>BQL</td>
</tr>
</tbody>
</table>

Table 7.—Individual concentrations (pg/ml) of r-metHuGDNF in cerebrospinal fluid of animals in the low- (15 µg/day) and mid-dose (30 µg/day) groups

<table>
<thead>
<tr>
<th>Sex</th>
<th>Group</th>
<th>End of First Month of Treatment Period</th>
<th>End of 6-Month Treatment Period</th>
<th>End of 3-Month Recovery Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (010)</td>
<td>Low-Dose (15 µg/day)</td>
<td>BQL</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Male (012)</td>
<td>End of Treatment</td>
<td>207</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Male (013)</td>
<td>Male (014)</td>
<td>1458</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Male (015)</td>
<td>Female (046)</td>
<td>812</td>
<td>NS</td>
<td>NA</td>
</tr>
<tr>
<td>Female (047)</td>
<td>End of Recovery</td>
<td>345</td>
<td>154</td>
<td>NA</td>
</tr>
<tr>
<td>Female (048)</td>
<td>Male (006)</td>
<td>BQL</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Female (049)</td>
<td>Female (050)</td>
<td>111</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Female (051)</td>
<td>End of Recovery</td>
<td>369</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Female (052)</td>
<td>Male (016)</td>
<td>183</td>
<td>BQL</td>
<td>NA</td>
</tr>
<tr>
<td>Female (053)</td>
<td>Male (017)</td>
<td>434</td>
<td>BQL</td>
<td>BQL</td>
</tr>
<tr>
<td>Female (054)</td>
<td>Female (055)</td>
<td>374</td>
<td>125</td>
<td>BQL</td>
</tr>
<tr>
<td>Female (056)</td>
<td>Female (057)</td>
<td>BQL</td>
<td>BQL</td>
<td>BQL</td>
</tr>
</tbody>
</table>

**“Affected” and “Unaffected” refer to animals with and without the cerebellar lesion, respectively. BQL = Below the limit of quantification. NS = No sample. NA = Not applicable. “End of Treatment” and “End of Recovery” refer to the scheduled necropsy interval.**

Table shows animals that survived to scheduled necropsy.

The pathogenesis of the cerebellar cortical lesion was not apparent, but because Purkinje cell loss was the only alteration in some areas, a primary loss of Purkinje cell neurons was suspected as the initial change. Atrophy of the molecular layer was believed to be associated with the Purkinje cell loss, as the molecular layer is composed largely of the extensive dendritic branches of Purkinje cells. Because the loss of granule cells was only seen in combination with the Purkinje cell and molecular layer effects, it was believed to be a secondary effect; however, the etiology is unclear.

Cerebellar Purkinje cells are vulnerable to multiple insults; consequently, their loss is common to many pathologic conditions (Fonnum and Lock, 2000), including global ischemia (Brierley and Excell, 1966; Brierley et al., 1969). Because ischemia can be produced by anesthesia-related hypotension or cardiac arrest (Zeiss et al., 2001; Yoshizawa et al., 2005), the possibility that a hypotensive event led to the lesions observed in the present study should be considered. However, all animals were exposed to similar anesthetic, surgical, and necropsy-related procedures, precluding unique treatment that might be expected to cause an ischemic event in a subset of animals, and no unexpected incidents that would be indicative of such an event were noted during surgery or other periods during which animals were under anesthesia.
Furthermore, the pattern of neuropathology associated with global ischemia typically includes damage to multiple regions of the brain, including the hippocampus, cerebral cortex, striatum (caudate and putamen), and cerebellum (Brierley and Excell, 1966; Brierley et al., 1969, 1980; Meldrum and Brierley, 1969; Kirino, 1982; Sato et al., 1990; Yoshida et al., 2002). The lesion in the present study was specific to the cerebellum; other areas of the brain known to be sensitive to ischemic damage were histologically unremarkable. Given these facts, available data suggests that the observed cerebellar lesions were produced by mechanisms other than global ischemia.

In considering potential underlying etiology, it is interesting to note the shared characteristics of the neuropathology produced by r-metHuGDNF and by the indole alkaloids, ibogaine and harmaline. When ibogaine or harmaline are administered systemically (IP) to rats, they produce ataxia and tremor that is associated with neuronal degeneration limited almost exclusively to the cerebellum (Singbartl et al., 1973; O’Hearn et al., 1993; O’Hearn and Molliver, 1993, 1999). Furthermore, within the cerebellum, the damage is specific to Purkinje cells and their dendrites (O’Hearn et al., 1993; O’Hearn and Molliver, 1993). Ibogaine and harmaline are believed to produce their effects on cerebellar Purkinje cells by excitotoxic mechanisms initiated by excessive activation of neurons in the inferior olive (O’Hearn et al., 1993; O’Hearn and Molliver, 1993, 1997). Cerebellar Purkinje cells are known to be exquisitely sensitive to excitatory amino acid-mediated damage (Garthwaite and Garthwaite, 1991; Brorson et al., 1995; Welsh et al., 2002; Sarna and Hawkes, 2003; Slemmer et al., 2005) because they receive extensive excitatory (glutamatergic) synaptic contact from climbing fibers from the inferior olive and from parallel fibers from granule cells (Llinas and Nicholson, 1976; Sugimori and Llinas, 1990; Brorson et al., 1995; O’Hearn and Molliver, 1997, 1999; Voogd and Glickstein, 1998; Welsh et al., 2002).

Purkinje cell loss produced in rats by ibogaine and harmaline administration, however, occurs bilaterally in discrete parasagittal bands, with the most pronounced effects apparent in the vermis (O’Hearn et al., 1993; O’Hearn and Molliver, 1993; Welsh et al., 2002). Such a pattern was not revealed in the present study. Furthermore, the lesion in the present study sometimes included pronounced hypocellularity in the granule cell layer, which has not been reported for other excitotoxicity-mediated cerebellar toxicity (Welsh et al., 2002). Therefore, consideration of an excitotoxic mechanism would require further investigation. It is also important to note that a common histologic appearance might only reflect the limited array of potential responses of the cerebellum to toxic insult, and not necessarily demonstrate a shared mechanism of action.

Excitotoxicity-mediated neuronal damage is circuitry-mediated, or trans-synaptic, meaning that the injury results from activation of existing neuronal pathways and the release of endogenous excitotoxic amino acids (O’Hearn and Molliver, 1993; O’Reilly et al., 2003), rather than through direct contact of the drug with the target site. Since direct functional connections between the putamen and cerebellum do not exist, and wider drug exposure occurred via the CSF in this study, the pathway by which r-metHuGDNF might have mediated an indirect excitotoxic response in the cerebellum is unknown.

Purkinje cells are the only efferent projections of the cerebellar cortex through their inhibitory (GABAergic) interaction with the deep cerebellar nuclei that project to upper motor neurons in the cortex (through the thalamus) and brainstem (Fonnum and Lock, 2000; Welsh et al., 2002; Sarna and Hawkes, 2003). Because the cerebellum modulates movement via this efferent pathway, the loss of Purkinje cells should be considered a functional deficit. Cerebellar damage is known to be associated with abnormalities in gait and posture, limb movement deficits (including ataxia and intention tremor), dysarthria, and oculomotor disturbances across species (Gembä et al., 1980; Trouillas et al., 1997; Fonnum and Lock, 2000; Sarna and Hawkes, 2003). In the present study, no observations of this nature were noted in affected animals. However, the extent of the observed cerebellar damage was generally limited (at least in 3 of the 4 affected animals), and the study was not designed to assess subtle changes in motor function.

The r-metHuGDNF-associated cerebellar damage warrants concern. First, the observed neuronal loss, by its very nature, would not be expected to be reversible upon cessation of treatment. Furthermore, the lesion was not apparent in MRI scans performed during the treatment or recovery periods, or by any of the other non-terminal evaluations used on the study. Consequently, the finding was not monitorable by non-invasive techniques. Finally, the occurrence of cerebellar lesions appears to be specific to the particular design parameters used in this study (e.g., route, regimen, duration of treatment, and/or recovery period), as the finding has not been observed in previous nonclinical studies evaluating r-metHuGDNF. Precisely how these design variables contributed to the r-metHuGDNF-induced cerebellar damage is not clear. Because of the limited data available from this study and the critical implications of the observed r-metHuGDNF-associated cerebellar neuronal loss, further studies directed at elucidating the cause and pathogenesis of the lesion are warranted.

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