Transport of Human Recombinant Brain-Derived Neurotrophic Factor (BDNF) Through the Rat Blood–Brain Barrier *in Vivo* Using Vector-Mediated Peptide Drug Delivery

William M. Pardridge,[1,2] Young-Sook Kang,[1] and Jody L. Buciak[1]

Received October 7, 1993; accepted November 29, 1993

The blood–brain barrier (BBB) transport of brain-derived neurotrophic factor (BDNF) in anesthetized rats was examined in the present studies using vector-mediated peptide drug delivery. Following tritiation, the BDNF was biotinylated via a disulfide linker and was coupled to a covalent conjugate of neutral avidin (NLA), which binds the biotinylated peptide with a high affinity, and the murine OX26 monoclonal antibody to the rat transferrin receptor. Owing to the abundance of transferrin receptors on brain capillary endothelium, the OX26 monoclonal antibody undergoes receptor-mediated transcytosis through the BBB, and the NLA–OX26 conjugate transports biotinylated peptide therapeutics through the BBB. The present studies show that while unconjugated BDNF was not transported through the BBB *in vivo*, the conjugation of biotinylated BDNF to the NLA-OX26 vector resulted in a marked increase in the brain delivery of BDNF, as defined by measurements of the percentage of the injected dose (ID) delivered per gram of brain. Although BDNF was not transported through the BBB *in vivo*, this cationic peptide was avidly bound by isolated human brain capillaries via a low-affinity, high-capacity system that was inhibited by protamine and by serum protein binding of BDNF. In conclusion, these studies show that the delivery of unconjugated BDNF to brain is nil owing to the combined effects of negligible BBB transport and rapid systemic clearance of intravenous administered BDNF. The brain delivery of BDNF may be augmented by conjugation of BDNF to BBB drug delivery vectors, such as the NLA–OX26 conjugate.

KEY WORDS: endothelium; transferrin receptor; avidin; liver; transcytosis.

[1] Department of Medicine, Brain Research Institute, UCLA School of Medicine, Los Angeles, California 90024.

[2] To whom correspondence should be addressed at Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024.

[3] *Abbreviations used:* BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; RSA, rat serum albumin; NSP, *N*-succinimidyl propionate; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido) ethyl-1,3′-dithiopropionate; RHB, Ringer Hepes buffer; PBHS, phosphate buffer, high saline; TCA, trichloroacetic acid; V_D, volume of distribution; ID, injected dose; D, dose; V_{ss}, steady-state volume of distribution; AUC, area under the curve; Cl, systemic clearance; PS, permeability–surface area product; V_o, plasma volume of distribution; NLA, neutral avidin; NGF, nerve growth factor; bio-BDNF, biotinylated BDNF; K_D, binding dissociation constant; B_{max}, maximal binding capacity; NSB, nonspecific binding; ED_{50}, concentration that causes 50% inhibition of binding; HSA, human serum albumin.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF)[3] is a member of the nerve growth factor (NGF) family (1,2) and, like NGF, may have beneficial effects as a peptide-based therapeutic for the treatment of neurodegenerative diseases of brain. BDNF is a trophic factor for cholinergic or dopaminergic neurons *in vitro* in tissue culture (3–6), is a trophic factor for dopaminergic neurons *in vivo* (7), and is a trophic factor for retinal rods and cones *in vivo* (8). BDNF gene expression is induced in a widespread manner in brain in response to either ischemia or hypoglycemia (9) and is expressed in the basal forebrain cholinergic system (10). Since BDNF is a potential neuropharmaceutical for the treatment of Alzheimer's disease or other degenerative or traumatic conditions of brain, it is useful to quantitate the extent to which this peptide is transported through the brain capillary endothelial wall, which makes up the blood–brain barrier (BBB) *in vivo*. Therefore, the present study was designed (a) to investigate whether BDNF is transported through the BBB and (b) to determine the extent to which BBB transport of BDNF may be augmented by the use of vector-mediated drug delivery. The latter involves the use of chimeric peptides, wherein a nontransportable peptide therapeutic, e.g., BDNF, is conjugated to a BBB drug delivery vector (11). Vectors include proteins that undergo either absorptive-mediated transcytosis through the BBB, e.g., cationized albumin (11), or receptor-mediated transcytosis through the BBB, e.g., a monoclonal antibody to the transferrin receptor (12). Conjugation of peptide-based therapeutics to brain drug delivery vectors is facilitated by the use of avidin–biotin technology (13). In this approach the peptide therapeutic is biotinylated via a disulfide linker and coupled to a conjugate of avidin and the BBB drug delivery vector. The activity of avidin/vector conjugates has recently been improved by the use of neutral forms of avidin, which increases the systemic bioavailability of the avidin/vector conjugate (14).

MATERIALS AND METHODS

Materials

Human recombinant BDNF was obtained from Amgen (Thousand Oaks, CA). The [^{3}H]*N*-succinimidyl propionate (NSP), 102 Ci/mmol, and the [1-^{14}C]acetic anhydride, 150 mCi/mmol, were purchased from Amersham Corp. (Arlington Heights, IL). The [^{14}C(U)]sucrose was purchased from Dupont-NEN (Boston, MA). Sulfosuccinimidyl 2-(biotinamido) ethyl-1,3′-dithiopropionate (NHS-SS-biotin) was purchased from Pierce Chemical Co. (Rockford, IL). Rat serum albumin (RSA), salmon protamine (grade 4), and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO). A 7.8 × 300-mm TSK-GEL G2000SW$_{XL}$ HPLC column was obtained from Toso Haas (Montgomeryville, PA).

Radiolabeling, Biotinylation, and Formation of Chimeric Peptides

Native RSA was labeled with [^{14}C]acetic anhydride to a specific activity of 530 mCi/mmol, as described previously

0724-8741/94/0500-0738$07.00/0

(15). The BDNF was tritiated with [^{3}H]NSP by transferring 5 mCi of [^{3}H]NSP (subsequent to evaporation of tolene diluent) to 500 μg of BDNF in 200 μL of 0.05 *M* Na_2HPO_4/0.15 *M* NaCl (PBS; pH 8.5), followed by capping and shaking at 4°C for 45 min. To this solution was added 250 μL of 0.2 *M* Na_2HPO_4 (pH 6.0)/0.4 *M* NaCl. The trichloroacetic acid (TCA) precipitability of this material was 72%, indicating that 0.92 lysine residue of BDNF monomer was ^{3}H-labeled, which is calculated from the ratio of the specific activity of [^{3}H]BDNF (91 Ci/mmol) to the specific activity of [^{3}H]NSP (102 Ci/mmol). The [^{3}H]BDNF eluted as a single peak from a 10 × 250-mm C4 reverse-phase high-performance liquid chromatography (HPLC) column at approximately 55% acetonitrile in 0.1% trifluoroacetic acid, following removal of TCA-soluble radioactivity via G-25 gel filtration.

Subsequent to the [^{3}H]NSP labeling, 50-μg aliquots of the [^{3}H]BDNF were biotinylated with a 10-fold molar excess of NHS-SS-biotin followed by shaking for 60 min at room temperature. The reaction was quenched by the addition of 1 μmol of glycine followed by shaking for an additional 30 min at room temperature. The 10-fold molar excess of NHS-SS-biotin, relative to the concentration of the BDNF monomer, was based on a molecular weight of 13,000 daltons for the BDNF monomer. Higher molar ratios of NHS-SS-biotin caused multibiotinylation and precipitation of the product. The volume of the [^{3}H]biotinylated-BDNF (bio-BDNF) solution was increased to 250 μL by the addition of 0.1 *M* Na_2HPO_4, 0.5 *M* NaCl, pH 6.9 (PBHS buffer), and 180 μg of a conjugate of neutral avidin (NLA) and the OX26 monoclonal antibody was added. The NLA-OX26 conjugate was prepared via a stable thioether linkage as described previously (14). A 200-μL volume of the [^{3}H]bio-BDNF/NLA-OX26 complex was purified by elution through a TSK G2000SW$_{XL}$ gel filtration HPLC column (7.8 mm × 30 cm). The column was eluted with PBHS isocratically at a flow rate of 0.5 mL/min and 0.5-mL fractions were collected. The column was monitored by measurement of both absorption at 280 nm and ^{3}H radioactivity. The [^{3}H]bio-BDNF/NLA-OX26 eluted at 6–7 mL and the ^{3}H radioactivity was greater than 99% TCA precipitable. The unconjugated [^{3}H]BDNF eluted at 9 mL. The [^{3}H]BDNF/NLA-OX26 chimeric peptide is comprised of the following components (16):

[^{3}H]-[BDNF]-S-S-[biotin]-[NLA]-S-[OX26]

BDNF Binding to Isolated Human Brain Capillaries

Capillaries were isolated from human autopsy brain as described previously (17). The binding of [^{3}H]BDNF to isolated human brain capillaries was measured at either 4 or 37°C in a final volume of 0.45 mL of Ringer Hepes buffer (RHB; pH 7.4) containing 0.1 g/dL human serum albumin (HSA). The duration of the binding assay was extended from 0.25 to 60 min. The concentration of unlabeled BDNF in the binding assay was varied from 0.5 to 25 μ*M*. The concentration of protamine ranged from 10 to 50 μg/mL and the concentration of human serum was varied from 20 to 80% serum as the final concentration. The mass of isolated human brain capillaries ranged from a total capillary protein content of 28 μg to 153 μg per tube. The portion of [^{3}H]BDNF binding to isolated human brain capillaries that was resistant to a mild acid wash was determined as described previously (17). The saturable binding of BDNF to isolated human brain capillaries was analyzed with a nonlinear regression analysis as described previously (16). Weighting the data gave essentially parameter estimates identical to those using unweighted data.

Pharmacokinetics and Brain Delivery of [^{3}H]BDNF *in Vivo*

Male Sprague–Dawley rats weighing 250–280 g were anesthetized with 100 mg/kg ketamine and 2 mg/kg xylazine ip. Initially, the pharmacokinetics and brain delivery of unconjugated [^{3}H]BDNF were determined. In these experiments, 0.2-mL solutions of RHB containing 0.1% BSA, 100 μCi/mL [^{3}H]BDNF (99% TCA precipitable), and 20 μCi/mL [^{14}C]RSA were injected into a femoral vein. The dose of [^{3}H]BDNF administered was 3 μg/rat or 12 μg/kg. Blood samples (0.25 mL) were collected via a PE50 catheter from the femoral artery at 0.25, 1, 2, 5, 15, 30, 60, 90, 120, and 180 min after injection. After blood sampling, the blood volume was replaced with the same volume of normal saline, and the plasma was separated by centrifugation. At 1, 2, or 3 hr after injection, the brain and four other organs (liver, kidney, heart, lung) were removed following decapitation. The TCA precipitability of both plasma and brain was determined. One milliliter of cold 10% TCA was added to 50 μL of plasma, followed by centrifugation. Brain TCA precipitability was measured by homogenizing a portion of brain tissue in 6 vol of cold 10% TCA followed by centrifugation.

Pharmacokinetic parameters were calculated by fitting plasma TCA-precipitable radioactivity data to a biexponential equation, i.e.,

$$A(t) = A_1 e^{-K_1 t} + A_2 e^{-K_2 t}$$

where $A(t)$ is the percentage ID per milliliter of plasma radioactivity, and ID is the injected dose. Plasma data were fit to the biexponential equation using a derivative-free nonlinear regression analysis (PARBMDP, Biomedical Computer P-Series developed at UCLA Health Sciences Computing Facilities). The data were weighted using either weight = 1/(concentration)^2 or weight = 1, where concentration is disintegrations per minute per microliter or percentage ID per milliliter. The brain volume of distribution (V_D) of [^{3}H]BDNF at 1, 2, and 3 hr after iv injection was determined from the ratio of the disintegrations per minute per gram of tissue to the disintegrations per minute per microliter of terminal plasma. Similarly, the V_D of [^{3}H]BDNF in liver, heart, lung, and kidney was also determined. The plasma clearance (Cl), the steady-state volume of distribution (V_{ss}), and the area under the plasma concentration curve (AUC) were calculated from the pharmacokinetic parameters as follows (18):

$$V_{ss} = \frac{D\,[(A_1/K_1^2) + (A_2/K_2^2)]}{AUC^2}$$

$$\text{AUC} = \frac{A_1}{K_1} + \frac{A_2}{K_2}$$

$$\text{Cl} = \frac{D}{\text{AUC}}$$

where D is the injected dose. The BBB permeability–surface area (PS) product, which is equivalent to the rate of organ clearance (μL/min/g) of the [^{3}H]BDNF in the brain or the four other organs, was determined as follows:

$$PS = \frac{[V_D - V_0]\, C_p(T)}{\int_0^t C_p(t)\, dt}$$

where $C_p(T)$ is the terminal plasma concentration and V_0 is the organ volume of distribution of the [^{14}C]RSA plasma volume marker. The brain delivery, or percentage ID per gram of brain, at a given time (t) after iv injection is

$$\%\ \text{ID g}^{-1}(t) = PS \times \text{AUC}(t)$$

$$\text{AUC}(t) = \int_0^t C_p(t)\, dt$$

The pharmacokinetic analysis and measurement of the brain delivery were then repeated with iv injection of 8 μCi/rat (equivalent to 1.2 μg BDNF/rat) of the [^{3}H]bio-BDNF/NLA-OX26 that was purified through the TSK column as described above. In these experiments, pharmacokinetics was measured over a time period of 120 min and the brain was removed at 60 and 120 min for evaluation of brain delivery of the [^{3}H]BDNF chimeric peptide. The pharmacokinetic parameters were then calculated as described above. In these studies, aliquots of plasma were removed at 15 and 60 min following iv injection and 200-μL aliquots of plasma pooled from three rats were injected onto the TSK gel filtration HPLC column, followed by elution in the PBHS buffer. These studies measured the extent to which the BDNF chimeric peptide was cleaved to unconjugated BDNF due to reduction of the disulfide linker between the biotin moiety and the BDNF.

Internal Carotid Artery Perfusion/Capillary Depletion Technique

Owing to the rapid metabolism of systemically administered [^{3}H]BDNF (Results), it was desirable to study the BBB transport of BDNF using experimental conditions that eliminated systemic metabolism. Therefore, [^{3}H]BDNF (4 μCi/mL) and [^{14}C]sucrose (1 μCi/mL) were infused into the internal carotid artery of anesthetized rats for a 5-min period using the *in situ* internal carotid artery perfusion technique described previously (16). The brain volume of distribution (V_D) of the [^{3}H]BDNF and [^{14}C]sucrose blood volume marker were determined from the ratio of disintegrations per minute per gram of tissue to disintegrations per minute per microliter of perfusate. Since the V_D value of [^{3}H]BDNF may exceed the V_D value of [^{14}C]sucrose owing to microvascular binding of the [^{3}H]BDNF, without any significant transport of [^{3}H]BDNF through the BBB, the brain homogenate was further analyzed with the capillary depletion technique, as described previously (16). This technique measures the V_D of either [^{3}H]BDNF or [^{14}C]sucrose in the total-brain homogenate, postvascular supernatant, and vascular pellet. The postvascular supernatant V_D is a measure of the transcytosis of [^{3}H]BDNF through the brain microvascular endothelium and into the brain interstitial space. The TCA precipitability of [^{3}H]BDNF in the perfusate both before and after perfusion and in the postvascular supernatant following perfusion was measured.

RESULTS

The unconjugated [^{3}H]BDNF bound avidly to isolated human brain capillaries and the binding was linear with respect to mass of capillary added to the individual tubes (Fig. 1). The binding was time and temperature dependent (Fig. 2), was saturable with both unlabeled BDNF (Fig. 1) and unlabeled protamine (Fig. 2), and was inhibited by increasing amounts of human serum (Fig. 3). The saturable BDNF binding was analyzed by nonlinear regression analysis (Materials and Methods) to yield a K_D of 0.50 ± 0.19 μM, a B_{max} = 1.1 ± 0.3 nmol/mg protein, and a nonspecific binding (NSB) of 9.1 ± 3.0% (Fig. 1). Virtually all of the BDNF binding to isolated capillaries was removed by a mild acid wash, indicating that little, if any, of the BDNF was internalized by the brain microvessels. The concentration of protamine which inhibited BDNF binding to brain capillaries was approximately 20 μg/mL (Fig. 2), and the concentration of human serum that caused 50% inhibition of binding was 20% serum (Fig. 3).

The [^{3}H]BDNF was not significantly degraded by either isolated human brain capillaries or human serum, as the TCA precipitability was 99 ± 1% following a 30-min incubation at 37°C with 50% human serum in either the presence or the absence of isolated human brain capillaries.

The [^{3}H]bio-BDNF/NLA-OX26 eluted through the TSK gel filtration HPLC column as a single peak at an elution

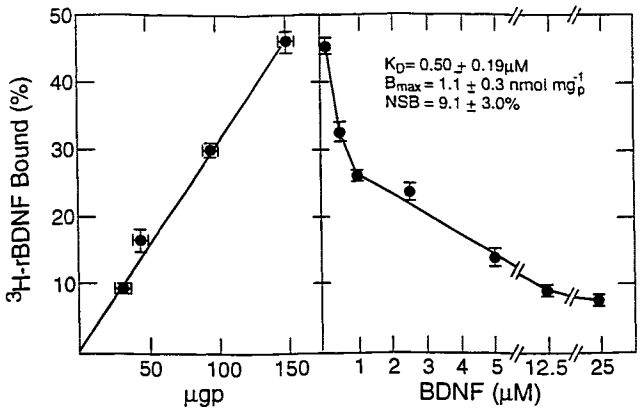

Fig. 1. The binding of [^{3}H]BDNF to isolated human brain capillaries is increased by the mass of capillaries per incubation tube (left) and is decreased by the addition of unlabeled BDNF to the incubation tube (right). The saturation data were analyzed by nonlinear regression analysis using a single saturable binding site and a nonspecific binding (NSB) binding site to yield the dissociation constant (K_D) and the maximum binding (B_{max}) of BDNF binding to human brain capillaries as shown. Data are mean ± SE (n = 3). [^{3}H]BDNF binding was 1–3% if no capillaries were added to the tubes.

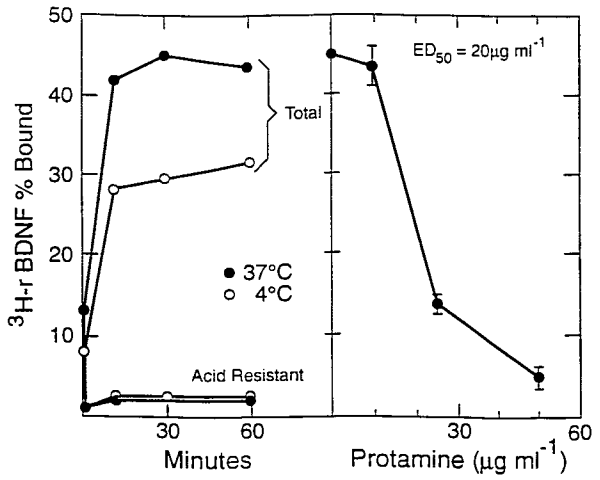

Fig. 2. The binding of [^{3}H]BDNF to isolated human brain capillaries is a function of time and temperature (left) and is inhibited by increasing concentrations of protamine (right). Virtually all of the BDNF binding at either 37 or 4°C was stripped by a mild acid wash from the brain capillaries. The protamine inhibition ED_{50} = 20 μg/mL. Data are mean ± SE (n = 3).

volume of 6–7 mL (Fig. 4). The peak of [^{3}H]radioactivity comigrated exactly with the A_{280} peak, which monitors the elution of the NLA-OX26 conjugate. The unconjugated [^{3}H]BDNF eluted through the column at a volume of approximately 9 mL (Fig. 4). When the [^{3}H]bio-BDNF/NLA-OX26 conjugate was injected with a biotin loading, approximately 80% of the [^{3}H]radioactivity shifted to an elution volume corresponding to unconjugated [^{3}H]BDNF (Fig. 4). These data provide evidence that the [^{3}H]BDNF is conjugated to the NLA-OX26 via the biotin linker. The disulfide linker could not be cleaved with dithiothreitol (DTT), as this reagent was found to denature the BDNF.

The clearance from the plasma compartment of either [^{14}C]RSA, [^{3}H]BDNF, or [^{3}H]bio-BDNF/NLA-OX26 is shown in Fig. 5 for up to 60 min after iv injection. The plasma TCA precipitability of the three proteins is shown in Table I. Multiplication of the total plasma radioactivity (dpm/

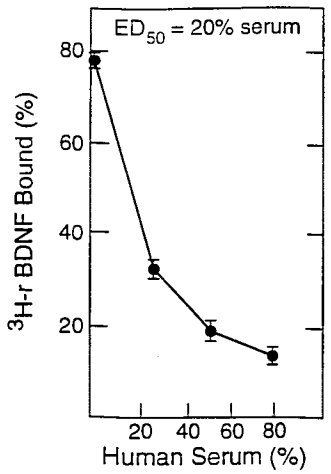

Fig. 3. The binding of [^{3}H]BDNF to isolated human brain capillaries is inhibited by increasing concentrations of serum with an ED_{50} of 20% serum. Data are mean ± SE (n = 3).

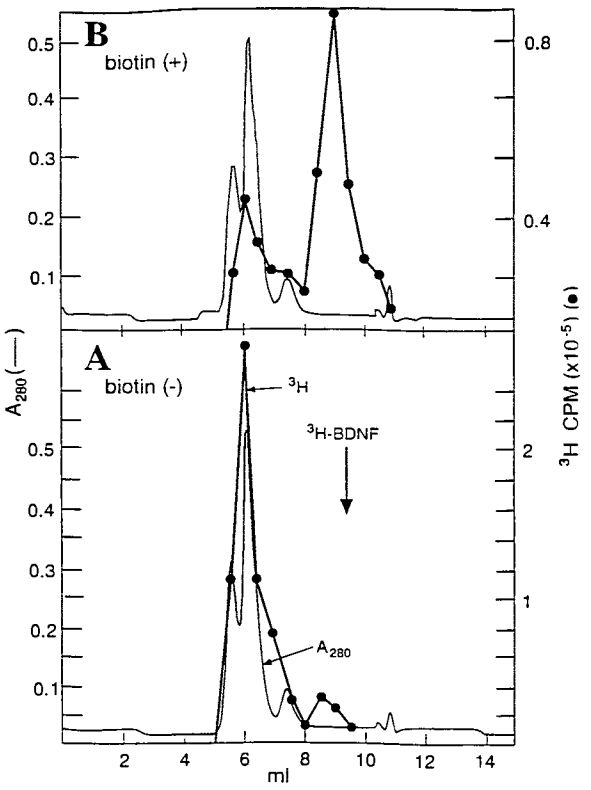

Fig. 4. Gel filtration HPLC of [^{3}H]bio-BDNF/NLA-OX26 in either the absence [biotin (−)] or the presence [biotin (+)] of an excess of unlabeled biotin. Both ^{3}H radioactivity and A_{280} were monitored for each fraction. The [^{3}H]bio-BDNF/NLA-OX26 migrated at an elution volume of 6 mL and unconjugated [^{3}H]BDNF migrated at an elution volume of 9 mL. In the absence of biotin, the [^{3}H]bio-BDNF comigrated with the NLA-OX26 antibody (which was detected by A_{280} absorbance). However, 80% of the radioactivity shifted to an elution volume that comigrated with unconjugated BDNF in the presence of a molar excess of unlabeled biotin. The injectate included 1 nmol NLA-OX26, 0.1 nmol [^{3}H]bioBDNF, and 25 nmol unlabeled biotin. The biotin was added to the NLA-OX26 conjugate 30 min before the addition of the [^{3}H]bioBDNF.

μL) by the fractional TCA precipitability yielded the plasma TCA-precipitable radioactivity, which is plotted in Fig. 5. The [^{14}C]RSA distributed with a half-time of 5.7 ± 3.9 min (Table II) and then was eliminated from the plasma compartment slowly, with a half-time of 6.4 ± 0.6 hr (Table II). Conversely, [^{3}H]BDNF distributed rapidly, with a half-time of 25 ± 4 sec (Table II), and was eliminated rapidly, with a half-time of 2.7 ± 0.1 min (Table II). Conjugation of the [^{3}H]BDNF through a biotin linker to the NLA-OX26 slowed the rate of egress of the [^{3}H]BDNF from the plasma compartment (Fig. 5) and increased the elimination half-time to 12.1 ± 0.9 min (Table II). Both [^{3}H]BDNF and [^{3}H]bio-BDNF/NLA-OX26 had a V_{ss} approximately double that of the [^{14}C]RSA (Table II). The Cl of the [^{3}H]BDNF was more than 280-fold faster than the Cl of the [^{14}C]RSA and was 4-fold faster than the systemic clearance of the [^{3}H]bio-BDNF/NLA-OX26 (Table II). Elution through the gel filtration TSK column of plasma obtained 15 min after conjugate administration to rats yielded a single peak of [^{3}H]radioactivity that eluted at 6–6.5 mL, i.e., the elution volume of the

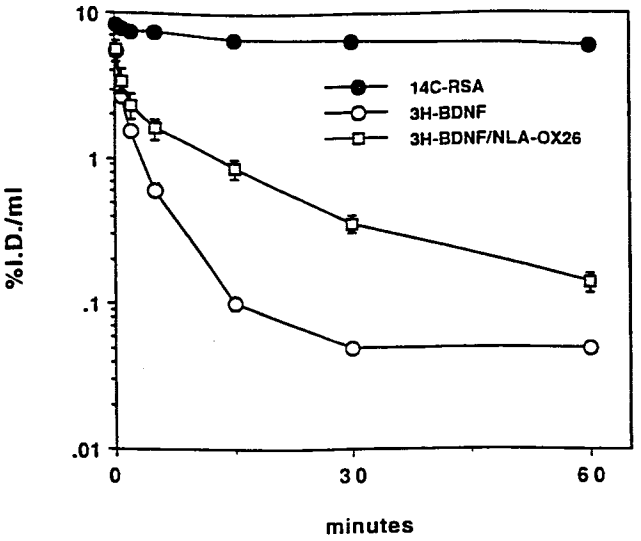

Fig. 5. The percentage ID per milliliter in plasma of [^{14}C]RSA, [^{3}H]BDNF, and [^{3}H]bio-BDNF/NLA-OX26 is shown for the first 60 min following iv administration of the three isotopes. Data are mean ± SE (n = 3 rats per point). Only TCA-precipitable radioactivity is shown.

conjugated [^{3}H]bio-BDNF/NLA-OX26 (Fig. 4). The plasma radioactivity at 60 min was too low to analyze.

The rapid systemic clearance of [^{3}H]BDNF is caused by high organ clearance by the kidney, lung, and liver, which cleared from 2.3 ± 0.8% ID/g (kidney) to 6.4 ± 2.2% ID/g (liver) within 60 min following an iv injection (Table III). Since the liver weighs approximately 10 g in a 280-g rat, 64% of the total injected [^{3}H]BDNF was cleared by the liver within 60 min following iv injection. The individual organ clearance estimates are given in Table III for heart, kidney, lung, and liver.

The brain uptake of [^{3}H]BDNF, either in its unconjugated form or in its biotinylated form conjugated to NLA-OX26, was measured following correction for both plasma and brain TCA precipitability. The V_D values for [^{3}H]BDNF and [^{3}H]bio-BDNF/NLA-OX26 are shown in Table II, and the BBB PS product and the percentage ID per gram values are given in Fig. 6. The brain delivery of [^{3}H]BDNF was low, at 0.0080 ± 0.0021% ID/g brain, and this delivery was increased ninefold when the [^{3}H]bio-BDNF was conjugated to NLA-OX26 (Fig. 6).

Table I. TCA Precipitability of [^{14}C]RSA, [^{3}H]BDNF, and [^{3}H]Bio-BDNF/NLA-OX26 in Plasma and Brain Following iv Administration in Anesthetized Rats[a]

Time (min)	[^{14}C]RSA		[^{3}H]BDNF		[^{3}H]bio-BDNF/NLA-OX26	
	Plasma	Brain	Plasma	Brain	Plasma	Brain
0.25	94 ± 1		91 ± 2		100	
1	98 ± 1		93 ± 1		100	
2	95 ± 1		85 ± 4		100	
5	94 ± 1		88 ± 2		100	
15	97 ± 1		88 ± 2		100	
30	96 ± 1		38 ± 3		65 ± 6	
60	96 ± 3	97 ± 3	20 ± 2	35 ± 14	29 ± 2	64 ± 9
90	95 ± 2		18 ± 1		20 ± 2	
120	97 ± 1	98 ± 2	15 ± 0	44 ± 18	16 ± 1	40 ± 2
180	98 ± 1	94 ± 3	20 ± 3	38 ± 17		

[a] Data are mean ± SE (n = 3).

The pharmacokinetic analysis and measurement of plasma TCA precipitability (Table I) showed that [^{3}H]BDNF was rapidly degraded following iv injection, which results in the release of TCA-soluble low molecular weight metabolites, which may subsequently undergo transport into brain, and other organs. Therefore, the PS product of [^{3}H]BDNF of 0.67 ± 0.14 μL/min/g given in Fig. 6 is an upper-limit value, and a major portion of this PS product may reflect artifactual uptake of TCA soluble ^{3}H-metabolites. To eliminate metabolism artifacts, the BBB transport of [^{3}H]BDNF was measured with the internal carotid artery perfusion/capillary depletion technique and these data are shown in Table IV. In corroboration of the isolated brain capillary experiments, [^{3}H]BDNF was rapidly bound by brain microvessels *in vivo* and achieved a V_D of 42 ± 15 μL/g after a 5-min perfusion. The V_D value was nearly fourfold greater than the V_D of the [^{14}C]sucrose plasma volume marker (Table IV). However, virtually all of this brain uptake represented binding to the vascular compartment; the postvascular supernatant V_D value of [^{3}H]BDNF was 14 ± 4 μL/g and this value was not statistically different from the V_D of [^{14}C]sucrose, which was 11 ± 1 μL/g. The perfusate and the postvascular supernatant TCA precipitability was greater than 99% following the 5-min internal carotid artery perfusion. These data indicate that although [^{3}H]BDNF achieves a brain volume of distribution considerably in excess of the plasma volume, this uptake represents binding to the microvasculature and no transport of BDNF through the BBB is measurable.

DISCUSSION

The results of the present studies are consistent with the following conclusions. First, BDNF avidly binds brain capillaries *in vitro* (Figs. 1–3) and *in vivo* (Table IV) via a process that is saturable by BDNF (with a low affinity and a high capacity), that is competed by another polycationic protein, protamine, and that is inhibited by serum protein binding. Second, BDNF is not endocytosed by isolated brain capillaries *in vitro* (Fig. 2) and is not transcytosed through the BBB *in vivo* (Table IV). Therefore, the BBB PS product determined by iv administration of BDNF and shown in Fig. 6 is an upper limit of the PS value and reflects largely brain uptake of TCA-soluble [^{3}H]BDNF metabolites. Third, BDNF is rapidly cleared from the plasma compartment *in vivo*, with a distribution $t_{1/2}$ of 25 ± 4 sec and an elimination $t_{1/2}$ of 2.7 ± 0.1 min (Table II). This rapid systemic clearance is due principally to avid uptake by liver and, to a lesser extent, to uptake by lung and kidney (Table III). Fourth, BDNF is rapidly degraded following systemic administration and this degradation occurs in tissue compartments, since no degradation of [^{3}H]BDNF is detectable by *in vitro* incubation with human serum (Results). Fifth, the brain delivery of BDNF is enhanced at least ninefold by coupling of biotinylated BDNF to a NLA-OX26 brain drug delivery vector. Sixth, the enhanced brain delivery of BDNF following bio-

Table II. Pharmacokinetic Parameters[a]

Parameter	[^{14}C]RSA	[^{3}H]BDNF	[^{3}H]Bio-BDNF/NLA-OX26
K_1 (min^{-1})	0.27 ± 0.11	1.75 ± 0.26	1.22 ± 0.11
K_2 (min^{-1})	0.0018 ± 0.0002	0.26 ± 0.01	0.0579 ± 0.0049
A_1 (% ID/mL)	1.80 ± 0.23	5.28 ± 0.60	4.70 ± 0.64
A_2 (% ID/mL)	5.53 ± 0.30	2.26 ± 0.32	2.11 ± 0.37
AUC (% ID · min/mL)	3043 ± 136	11.8 ± 0.8	40.4 ± 5.8
V (mL/kg)	70.9 ± 3.4	113 ± 7	150 ± 29
Cl_{ss} (mL/min/kg)	0.13 ± 0.01	37.0 ± 2.5	9.34 ± 1.49
$t_{1/2}^{1}$ (min)	5.74 ± 3.88	0.42 ± 0.07	0.58 ± 0.06
$t_{1/2}^{2}$ (min)	382 ± 32	2.72 ± 0.08	12.1 ± 0.9
V_D (μL/g)	13 ± 1	148 ± 27	498 ± 87

[a] All parameters are determined from TCA-corrected measurements of plasma and brain radioactivity; plasma measurements were extended to 180 min for [^{14}C]RSA and to 60 min for [^{3}H] BDNF and [^{3}H]bio-BDNF/NLA-OX26.

tinylation and coupling to the NLA-OX26 vector is due to dual effects of the vector: (a) enhanced systemic bioavailability, as reflected in the increased plasma AUC of [^{3}H]bio-BDNF/NLA-OX26 compared to [^{3}H]BDNF, and (b) increased BBB PS product of BDNF following biotinylation and coupling to the NLA-OX26 vector (Fig. 6).

BDNF, like its related molecules, NGF and neurotrophin-3, is a polycationic protein with a preponderance of lysine and arginine residues relative to glutamate and aspartate moieties (19–21). Previous studies have shown that some polycationic proteins, such as cationized albumin, undergo absorptive-mediated binding and endocytosis into isolated brain capillaries *in vitro* and absorptive-mediated transcytosis through the BBB *in vivo* (11). The binding of polycationic proteins to isolated microvessels *in vitro* may occur on either the lumenal endothelial membrane, which has an abundance of sialic acid negative charges (22), or on the ablumenal membrane, which has a majority of heparan sulfate negative charges (22). The absorptive-mediated binding of BDNF, like that of cationized albumin, is competitively inhibited by protamine and the ED_{50} (20 μg/mL; Figure 2) of protamine inhibition of BDNF binding to isolated human brain capillaries is comparable to the ED_{50} of protamine inhibition of cationized albumin binding to isolated bovine brain capillaries (11). Aside from protamine inhibition, another characteristic of absorptive-mediated binding to isolated brain capillaries is the relatively low affinity and high capacity; BDNF binding to isolated human brain capillaries has a K_D of 0.50 ± 0.19 μM and a very high B_{max} of 1.1 ± 0.3 nmol/mg$_p$. The B_{max} of BDNF binding to isolated human brain capillaries is more than 5000-fold higher than the B_{max} of insulin, insulin-like growth factor (IGF), or transferrin binding to isolated human brain capillaries, which is a receptor-mediated process (11). Therefore, the saturable binding of BDNF to brain capillaries is likely due to the cationic charge of this protein rather than to the affinity of BDNF for a specific receptor on human brain capillaries.

Table III. Organ Clearance and Delivery of [^{3}H]BDNF[a]

Organ	[^{14}C]RSA V_o (μL/g)	[^{3}H]BDNF Organ clearance (μL/min/g)	[^{3}H]BDNF Uptake (% ID/g)
Heart	150 ± 16	13 ± 2	0.15 ± 0.02
Kidney	131 ± 25	194 ± 62	2.3 ± 0.8
Lung	225 ± 17	203 ± 12	2.4 ± 0.2
Liver	119 ± 48	538 ± 177	6.4 ± 2.2

[a] Mean ± SE (n = 3). Measurements made 60 min after iv injection and calculated from TCA-precipitable radioactivity in plasma and total tissue radioactivity. V_o, plasma volume of distribution.

BDNF is apparently bound by serum proteins, as human serum inhibits BDNF binding to isolated human brain capillaries (Fig. 3). In this respect, BDNF shares properties similar to those of protamine, which avidly binds albumin and γ-globulins in serum (23). However, the serum binding of BDNF does not significantly retard the rapid egress of this protein from the plasma compartment following iv administration (Fig. 5). The rapid systemic clearance of BDNF is due principally to uptake by the liver, as the BDNF hepatic clearance, 538 ± 177 μL/min/g, is approximately 25% of the blood flow through that organ (24). Therefore, the first-pass extraction of BDNF by liver is at least 25%, and more than 60% of the total systemic BDNF clearance can be accounted for by liver alone during the first 60 min following iv administration (Results). The rapid organ clearance of BDNF by kidney is expected, since polycationic proteins, such as cationized rat albumin, are preferentially extracted by this organ *in vivo* (11). However, cationized rat albumin is not cleared by rat lung *in vivo;* indeed, native albumin is cleared by rat lung faster than is cationized rat albumin (11). Conversely, other highly cationic proteins such as BDNF (Results) or histone (11) are efficiently cleared across the pulmonary microvasculature. Subsequent to the clearance of BDNF from the plasma compartment by organs such as liver, lung, or kidney, there is rapid degradation of the protein as shown by the rate of decrease in plasma radioactivity that is TCA precipitable (Table I). This degradation may occur in the parenchymal compartment rather than the microvascular or plasma compartment, since [^{3}H]BDNF was metabolically stable following incubation at 37°C in human serum with or without human brain capillaries (Results).

Although BDNF is bound by brain capillaries either *in vitro* (Figs. 1–3) or *in vivo* (Table IV), BDNF is neither endocytosed by brain capillaries *in vitro* (Fig. 2) or transcy-

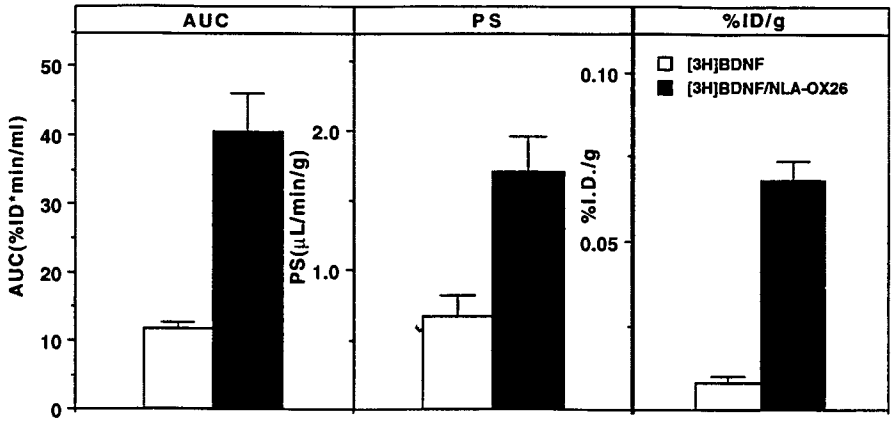

Fig. 6. The area under the plasma concentration curve (AUC), the BBB permeability–surface area (PS) product, and the percentage injected dose per gram brain (% ID/g) for [^{3}H]BDNF and [^{3}H]bio-BDNF/NLA-OX26 at 60 min after an iv injection. Mean ± SE (n = 3 rats each).

tosed *in vivo* (Table IV). Therefore, if BDNF is to be used as a neuropharmaceutical for the treatment of disorders of the brain or spinal cord, a brain drug delivery system may be utilized. Ideally, such a delivery system would have the dual effects of causing (a) an augmentation in the rate of transcytosis of BDNF through the BBB as reflected by an enhanced BBB PS product and (b) an increase in the systemic bioavailability of BDNF, as reflected by an increased plasma AUC. Enhanced brain delivery of BDNF was achieved in the present studies by coupling biotinylated BDNF to a covalent conjugate of NLA-OX26. Recent studies have shown that the biotinylation of a vasoactive intestinal peptide (VIP) analogue via a disulfide bridge, followed by subsequent coupling to a conjugate of avidin-OX26, enabled the VIP analogue to undergo transport through the BBB *in vivo* and to induce CNS pharmacologic effects, a 65% increase in cerebral blood flow (16). There was no measurable brain uptake or CNS pharmacologic effect of the VIP analogue if the peptide drug was administered systemically without the use of the BBB drug delivery vehicle (16). Other recent studies show a trophic effect of NGF on cholinergic neurons in neonatal explants of the anterior chamber of the eye, provided the NGF is coupled to a BBB drug delivery vector, such as the OX26 monoclonal antibody (12).

Conjugation of BDNF to the OX26 monoclonal antibody increases the brain V_D threefold (Table II). However, the V_D parameter must be normalized by the plasma AUC to reflect brain uptake quantitatively (see equations for % ID/g; Materials and Methods). When this is done, the brain uptake of BDNF is increased at least ninefold following biotinylation of the peptide and coupling to the NLA-OX26 vector (Fig. 6). The increase in brain delivery of BDNF caused by coupling to the vector is probably more than ninefold since the BBB PS product (and % ID/g) of nonconjugated BDNF is an overestimate owing to the rapid systemic clearance and degradation of BDNF following iv administration. There is conversion of 80% of the plasma radioactivity to TCA-soluble metabolites within 60 min following iv administration (Table I). These TCA-soluble metabolites may include [^{3}H]*N*-propyl lysine, which may undergo transport into brain via BBB amino acid transport systems (11). This rapid degradation of BDNF confounds interpretation of brain uptake data obtained following iv administration of the peptide. Therefore, we performed measurements of BBB transport of [^{3}H]BDNF following internal carotid artery perfusion coupled with the capillary depletion technique. This technique eliminates systemic degradation artifacts and also allows for a distinction between vascular sequestration of the labeled peptide and actual transcytosis into the brain parenchyma. These data (Table IV) provide evidence that BDNF is not transported through the BBB *in vivo*, although the peptide is bound by the microvasculature in brain. Therefore, the BBB transcytosis PS for BDNF is very low. Another factor contributing to the very low brain delivery of BDNF following iv administration (Table II) is the reduced plasma AUC of BDNF. For example, the plasma AUC of a plasma volume marker, such as [^{14}C]RSA, is 260-fold greater than the plasma AUC of [^{3}H]BDNF (Table II). One reason for the very high rate of systemic clearance of BDNF from the plasma compartment is the low dose (12 μg/kg; Materials and Methods) of peptide that was administered. Higher doses may saturate hepatic clearance of BDNF and this would reduce the systemic clearance and raise the plasma AUC and the $t_{1/2}$ of BDNF elimination from plasma.

The 60-min plasma AUC of [^{3}H]bio-BDNF/NLA-OX26, 40 ± 6% ID · min/mL, is increased more than threefold relative to the AUC of unconjugated BDNF alone (Table II). However, the plasma AUC of [^{3}H]bio-BDNF/NLA-OX26 is 10-fold lower than the corresponding plasma AUC of [^{3}H]biotin/NLA-OX26, 426 ± 43% ID · min/mL (14). Therefore,

Table IV. Brain Volume of Distribution (V_D) of [^{14}C]Sucrose and [^{3}H]BDNF Following a 5-min Internal Carotid Artery Infusion Followed by Capillary Depletion Analysis of Brain Homogenate[a]

Fraction	V_D (μL/g)	
	[^{14}C]Sucrose	[^{3}H]BDNF
Homogenate	11 ± 1	42 ± 15
Postvascular supernatant	11 ± 1	14 ± 4
Vascular pellet	0.63 ± 0.33	11 ± 5

[a] Mean ± SE (n = 3). [^{3}H]BDNF infused at a concentration of 4 μCi/mL (0.6 μg/mL or 43 n*M*).

conjugation of the cationic BDNF to the transport vector results in an increase in the systemic clearance of the transport vector. That is, the rate of systemic clearance of the [^{3}H]bio-BDNF/NLA-OX26 conjugate is closer to the rate of the systemic clearance of unconjugated BDNF than to the rate of systemic clearance of the [^{3}H]biotin/NLA-OX26 conjugate (14). The increased rate of systemic clearance of the OX26 monoclonal antibody caused by coupling of the cationic BDNF parallels previous studies showing that conjugation of the cationic protein, avidin, to the OX26 antibody increases the systemic clearance of the OX26 antibody. The enhanced rate of systemic clearance of the OX26 antibody caused by conjugation of the cationic avidin to the protein results in a decrease in the plasma AUC and a decrease in the brain delivery of the OX26 antibody (25). On the basis of these findings, subsequent studies evaluated the utility of using neutral forms of avidin to facilitate conjugation of biotinylated therapeutics to the OX26 antibody (14). These recent studies show that the plasma AUC of the NLA-OX26 conjugate is increased relative to the plasma AUC of the avidin-OX26 and approximates the plasma AUC of unconjugated OX26 antibody alone (14). Therefore, the use of neutral forms of avidin allows for the formation of avidin-OX26 conjugates that have improved systemic pharmacokinetic characteristics and allows for the production of universal brain drug delivery vectors that may transport into brain many different biotinylated therapeutics. The ability of the NLA-OX26 conjugate to achieve maximum brain delivery of peptide therapeutics, however, is proportional to the plasma bioavailability (AUC) of the vector, which may be altered by the conjugation of peptide therapeutics that are rapidly cleared by peripheral tissues. These considerations underscore the general principle that the ultimate delivery of peptide-based therapeutics to brain is a dual function of both BBB permeability properties (i.e., the transport vector) and the plasma AUC (i.e., the pharmacokinetics).

Finally, the present studies only test the feasibility of enhancing BDNF delivery to brain using vector-mediated peptide drug delivery systems, and do not address the pharmacodynamics of drug action in brain. Such investigations will require the demonstration of (a) cleavage of peptide from the vector in brain *in vivo,* (b) peptide binding to the appropriate receptor in brain, and (c) pharmacologic efficacy following systemic administration. The cleavage of disulfide-based chimeric peptides in brain is rapid and occurs within minutes of exposure to brain either *in vitro* (11) or *in vivo* (26). Regarding retention of receptor binding following conjugation of BDNF, it may be desirable in future studies to biotinylate the peptide via carboxyl moieties, as performed previously for NGF (12,27), rather than amino groups on the peptide. The pharmacologic effect in brain of BDNF chimeric peptides will require the delivery to brain of adequate concentrations of BDNF. In the present studies, 0.072% of the injected BDNF dose (3 μg) was delivered per g brain, which achieved a brain BDNF concentration of 2 ng/g brain. Higher brain concentrations, e.g., 1 μg/g brain, may be achieved with systemic administration of higher doses, e.g., 1.5 mg, of peptide. The administration of higher doses of peptide requires higher doses of vector, which may then saturate the BBB transport system, i.e., the transferrin receptor. However, previous studies have shown that the plasma AUC of the conjugate increases (owing to saturation of peripheral transferrin receptors) in proportion to the decrease in BBB PS product, resulting in minimal changes in the fractional delivery of drug to brain when large doses of vector are administered (25).

ACKNOWLEDGMENTS

The authors are indebted to Drs. James Miller and Robert Rosenfeld of Amgen, Inc., for valuable discussions and to Dr. Ulrich Bickel for review of the manuscript and for assistance in the nonlinear regression analyses. Sherri J. Chien skillfully prepared the manuscript. This work was supported by NIH Grant RO1-DA06748 and by Amgen Inc.

REFERENCES

1. W. C. Mobley, A. C. Server, D. N. Ishii, R. J. Riopelle, and E. M. Shooter. Nerve growth factor. *N. Engl. J. Med.* 297:1096–1104 (1977).
2. G. D. Yancopoulos, P. C. Maisonpierre, N. Y. Ip, T. H. Aldrich, L. Belluscio, T. G. Boulton, M. H. Cobb, S. P. Squinto, and M. E. Furth. Neurotrophic factors, their receptors, and the signal transduction pathways they activate. *Cold Spring Harbor Symp. Quant. Biol.* 55:371–379 (1990).
3. R. F. Alderson, A. L. Alterman, Y.-A. Barde, and R. M. Lindsay. Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. *Neuron* 5:297–306 (1990).
4. B. Knüsel, J. W. Winslow, A. Rosenthal, L. E. Burton, D. P. Seid, K. Nikolics, and F. Hefti. Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proc. Natl. Acad. Sci. USA* 88:961–965 (1991).
5. C. Hyman, M. Hofer, Y.-A. Barde, M. Juhasz, G. D. Yancopoulos, S. P. Squinto, and R. M. Lindsay. BDNF is a neurotrophic factor for dopaminergic neurons of thesubstantia nigra. *Nature* 350:320–232 (1991).
6. M. B. Spina, S. P. Squinto, J. Miller, R. M. Lindsay, and C. Hyman. Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. *J. Neurochem.* 59:99–106 (1992).
7. C. A. Altar, C. B. Boylan, C. Jackson, S. Hershenson, J. Miller, S. J. Wiegand, R. M. Lindsay, and C. Hyman. Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. *Proc. Natl. Acad. USA* 89:11347–11351 (1992).
8. M. M. LaVail, K. Unoki, D. Yasumura, M. T. Matthes, G. D. Yancopoulos, and R. H. Steinberg. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl. Acad. Sci. USA* 89:11249–11253 (1992).
9. O. Lindvall, P. Ernfors, J. Bengzon, Z. Kokaia, M.-L. Smith, B. K. Siesjö, and H. Persson. Differential regulation of mRNAs for nerve growth factor, brain-derived neurotrophic factor, and neurotrophin 3 in the adult rat brain following cerebral ischemia and hypoglycemic coma. *Proc. Natl. Acad. Sci. USA* 89:648–652 (1992).
10. H. S. Phillips, J. M. Hains, G. R. Laramee, A. Rosenthal, and J. W. Winslow. Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. *Science* 250:290–294 (1990).
11. W. M. Pardridge. *Peptide Drug Delivery to the Brain,* Raven Press, New York, 1991, pp. 1–357.
12. P. M. Friden, L. R. Walus, P. Watson, S. R. Doctrow, J. W. Kozarich, C. Backman, H. Bergman, B. Hoffer, F. Bloom, and A.-C. Granholm. Blood-brain barrier penetration and in vivo activity of an NGF conjugate. *Science* 259:373–377 (1993).
13. T. Yoshikawa and W. M. Pardridge. Biotin delivery to brain with a covalent conjugate of avidin and a monoclonal antibody to the

transferrin receptor. *J. Pharmacol. Exp. Ther.* **263**:897–903 (1992).
14. Y.-S. Kang and W. M. Pardridge. Use of neutral-avidin improves pharmacokinetics and brain delivery of biotin bound to an avidin-monoclonal antibody conjugate. *J. Pharmacol. Exp. Ther.* **269**:344–350 (1994).
15. W. M. Pardridge, J. L. Buciak, and T. Yoshikawa. Transport of recombinant CD4 through the rat blood-brain barrier. *J. Pharmacol. Exp. Ther.* **261**:1175–1180 (1992).
16. U. Bickel, T. Yoshikawa, E. M. Landaw, K. F. Faull, and W. M. Pardridge. Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery. *Proc. Natl. Acad. Sci.* **90**:2618–2622 (1993).
17. W. M. Pardridge, J. Eisenberg, and J. Yang. Human blood-brain barrier insulin receptor. *J. Neurochem.* 44:1771–1778 (1985).
18. M. Gibaldi and D. Perrier. *Pharmacokinetics*, Marcel Dekker, New York (1982).
19. J. Leibrock, F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.-A. Barde. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**:149–152 (1989).
20. K. R. Jones and L. F. Reichardt. Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc. Natl. Acad. Sci. USA* 87:8060–8064 (1990).
21. A. Rosenthal, D. V. Goeddel, T. Nguyen, E. Martin, L. E. Burton, A. Shih, G. R. Laramee, F. Wurm, A. Mason, K. Nikolics, and J. W. Winslow. Primary structure and biological activity of human brain-derived neurotrophic factor. *Endocrinology* 129:1289–1294 (1991).
22. A. W. Vorbrodt. Ultracytochemical characterization of anionic sites in the wall of brain capillaries. *J. Neurocytol.* 18:359–368 (1989).
23. W. M. Pardridge, J. L. Buciak, Y.-S. Kang, and R. J. Boado. Protamine-mediated transport of albumin into brain and other organs in the rat. Binding and endocytosis of protamine-albumin complex by microvascular endothelium. *J. Clin. Invest.* **92**: 2224–2229 (1993).
24. M. A. Lupo, W. T. Cefalu, and W. M. Pardridge. Kinetics of lactate transport into rat liver in vivo. *Metabolism* **39**:374–378 (1990).
25. Y.-S. Kang, U. Bickel, and W. M. Pardridge. Pharmacokinetics and saturable blood-brain barrier transport of biotin bound to a conjugate of avidin and a monoclonal antibody to the transferrin receptor. *Drug Metabol. Disp.* **22**:99–105 (1994).
26. U. Bickel and W. M. Pardridge. Rapid cleavage of disulfide-based chimeric peptides by rat brain in vivo. *Faseb J.*, in press (1994).
27. M. B. Rosenberg, E. Hawrot, and X. O. Breakefield. Receptor binding activities of biotinylated derivatives of β-nerve growth factor. *J. Neurochem.* **46**:641–648 (1986).