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PEGylation of brain-derived neurotrophic factor for preserved biological activity and enhanced spinal cord distribution

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Abstract

Brain-derived neurotrophic factor (BDNF) was covalently attached to polyethylene glycol (PEG) in order to enhance delivery to the spinal cord via the cerebrospinal fluid (intrathecal administration). By varying reaction conditions, mixtures of BDNF covalently attached to one (primary), two (secondary), three (tertiary) or more (higher order) PEG molecules were produced. The biological activity of each resulting conjugate mixture was assessed with the goal of identifying a relationship between the number of PEG molecules attached to BDNF and biological activity. A high degree of *in vitro* biological activity was maintained in mixtures enriched in primary and secondary conjugate products, while a substantial reduction in biological activity was observed in mixtures with tertiary and higher order conjugates. When a biologically active mixture of PEG-BDNF was administered intrathecally, it displayed a significantly improved half-life in the cerebrospinal fluid and an enhanced penetration into spinal cord tissue relative to native BDNF. Results from these studies suggest a PEGylation strategy that preserves the biological activity of the protein while also improving the half-life of the protein *in vivo*. Furthermore, PEGylation may be a promising approach for enhancing intrathecal delivery of therapeutic proteins with potential for treating disease and injury in the spinal cord.

Keywords

Intrathecal drug delivery; Brain-derived neurotrophic factor; PEGylation; Biological activity; Confocal microscopy

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Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of molecules and exhibits therapeutic benefits for several neurodegenerative diseases¹. BDNF can limit tissue damage after spinal cord injury², augment the function of spared neural systems³, promote neural repair and regeneration², and promote cell survival and neurite outgrowth⁴. BDNF also has potential as a therapeutic agent for neuropathic pain, as low doses of BDNF can suppress abnormal pain reactivity caused by peripheral nerve injury^{5,6}.

To treat disease and injury in the spinal cord, BDNF is commonly administered intrathecally via the cerebrospinal fluid (CSF) surrounding the spinal cord. Protein in the CSF then diffuses into spinal cord tissue to reach and impact cellular targets. However, in most cases, protein half-life (typically 1-3 hours)⁷ due to turn over in the CSF limits the time scale over which biologically active levels of BDNF can be maintained in the CSF. This in turn, limits the amount of BDNF that is capable of diffusing from the CSF into the parenchyma towards cellular targets. Once in tissue, the protein is also subject to elimination via binding to cell surface receptors and enzymatic degradation prior to reaching cellular targets, which further limits its therapeutic availability. To improve the efficacy of intrathecal protein administration, delivery strategies designed to increase the bioavailability of the protein in the CSF and in the parenchyma are necessary.

Covalent attachment of synthetic polymers to proteins has been shown to improve protein half-life and penetration into tissues^{1,2,8-24}. The polymer polyethylene glycol (PEG) is the most widely used for this purpose^{10,25-27}. While the attachment of PEG to a protein can sterically hinder the protein's access to receptors and subsequent protein biological activity, PEGylated proteins have an increased half-life in the bloodstream. The increased circulation time of the protein compensates for the reduction in activity and a therapeutic benefit is often observed when examined *in vivo*. While improvements in the half-life of PEGylated proteins *in vivo* often overcome *in vitro* biological activity losses^{21,28}, minimizing the loss in activity by controlling the PEGylation reaction would be advantageous. The resultant therapeutic would not only improve patient compliance, as the number of necessary injections could be reduced due to the longer circulation time of the protein, but it would be more cost-effective as lower dosages would be necessary to achieve therapeutic effects.

The focus of this work is to PEGylate BDNF in order to enhance *in vivo* properties after intrathecal administration while maintaining full *in vitro* biological activity, a feat that has only been reported with carboxyl-directed BDNF PEGylation²¹, but not previously reported with amine-directed BDNF PEGylation strategies^{1,2}. PEGylation of the N-terminus of proteins tends to retain the biological activity of proteins when characterized *in vitro*^{23,29}, including epidermal growth factor (EGF)¹². Based on success with this related growth factor, we pursued a PEGylation scheme that targets the N-terminus of BDNF. Because there tends to be an inverse relationship between the number of PEG molecules attached to a protein and its *in vivo* clearance rate²⁴, we also examined the functionality of BDNF when attached to two or more PEG molecules. Overall, our approach was to maximize the number of PEG molecules attached to the protein while maintaining the *in vitro* biological activity by minimizing the number of PEG molecules that attach to residues located within BDNF

functional sites. The effect of attaching one, two, three or more PEG molecules to BDNF on *in vitro* biological activity was examined. The mixture with the highest level of biological activity was identified and the improvement in half-life and penetration into the spinal cord parenchyma relative to unmodified BDNF following intrathecal administration was measured.

Materials and Methods

BDNF PEGylation with aldehyde chemistry

PEG conjugation with BDNF using aldehyde chemistry (mPEG-ButyrALD, Fig. 1a) was conducted in reaction buffer that consisted of 50 mM sodium phosphate with 100 mM sodium chloride at a pH of 6.3. 1.780 mg of rhBDNF (Amgen) was added to 1 ml of the reaction buffer as the protein mix. For a 60-fold PEG to BDNF dimer molar excess and a 60-fold reducing agent to BDNF dimer molar excess, 10 mg of mPEG-Butyrald-5000 (Nektar) was added to 0.6575 ml of the reaction buffer as the polymer mix and 1.72 mg of sodium cyanoborohydride (Sigma-Aldrich) was added to 10 ml of reaction buffer as the reducing agent mix. 125 μ l of the protein mix, 200 μ l of the polymer mix and 175 μ l of the reducing agent mix were combined in a polypropylene tube and agitated at room temperature for 24 hours. Reactions were stopped by transferring the products to -80°C until further analysis. Variations in the molar excess of constituents to BDNF were conducted by holding the concentration of BDNF constant.

BDNF PEGylation with NHS ester chemistry

NHS ester chemistry (mPEG-SPA, Fig. 1b) was also used to conjugate PEG with BDNF and this reaction was conducted in phosphate buffered saline at a pH of 7.4. 0.89 mg of BDNF was added to 1 ml of PBS as the protein mix. For a 15-fold PEG to BDNF dimer molar excess 2.4 mg of mPEG-SPA (Nektar) was added to 1 ml of the reaction buffer as the polymer mix. 250 μ l of the protein mix and 250 μ l of the polymer mix were combined in a polypropylene tube and agitated at room temperature for 24 hours. Reactions were stopped by transferring the products to -80°C until further analysis.

Mass spec analysis

Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager-DE STR, Perkin Elmer) was used to obtain mass information for PEG, BDNF and representative PEG-BDNF conjugate mixtures. 0.5 μ l samples were co-crystallized with matrix (sinapinic acid, Agilent) on gold-coated sample plates. Data were summed over 100 acquisitions in delayed linear extraction mode with a 25 kV accelerating voltage, a 50 V guide wire voltage and a 300 ns delay.

Gel electrophoresis and immunoblotting

Reaction products were analyzed by SDS-PAGE using 10% pre-cast gels according to manufacturer recommended reagents and protocols (Bio-Rad). Coomassie staining (Bio-Rad) was conducted with gels that had been loaded with 8.9 μ g of total protein. Immunoblotting with OPTI-4CN detection was also conducted according to manufacturer recommended reagents and protocols (Bio-Rad). 500 ng of total protein was loaded for

detection with 1:2000 diluted rabbit-anti-BDNF polyclonal antibody (Chemicon Ab1779) as the primary antibody and 1:3000 goat-anti-rabbit-HRP conjugate (Bio-Rad) as the secondary antibody.

Band density analyses were conducted on coomassie stained gels with NIH ImageJ software. Image look up tables were inverted and the products of the mean and area measurements were taken on a black and white scale using a polygonal fit around each observed band. The product of the mean and area measurement was measured as the intensity value for each species. The total intensity for all bands in a conjugate mixture was determined and the fractional intensity of the free BDNF band was multiplied by the total protein concentration to estimate the residual free BDNF concentration.

In vitro biological activity assay

The *in vitro* biological activity assay was conducted using a rat pheochromocytoma cell line (PC12, passage number 13-18) that stably expresses the trkB receptor for BDNF. Cells were grown in RPMI medium supplemented with 10% horse serum, 5% fetal bovine serum and 1% penicillin-streptomycin (all from Invitrogen) in 24-well collagen coated plates (8 µg/cm², Vitrogen-100, Angiotech). 24 hours after seeding the plates at a density of 1.0×10^6 cells/ml, the cells were incubated with 0.5 ng/ml of BDNF or PEG-BDNF conjugate mixtures for an additional 24 hours. The medium was removed by aspiration and the cells were fixed onto the plates with 4% paraformaldehyde. The number of cells extending neurites longer than two cell bodies was then assessed and error values were represented as standard error of the mean. For each condition 40 different groups consisting of 10 cells each were analyzed to determine the mean fraction of neurite extension.

Intrathecal injections

All animal procedures were in accordance with the Institutional Animal Care and Use Committee at the University of Colorado at Boulder and NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were also observed. Pathogen-free adult male Sprague-Dawley rats were used in all experiments. Rats (250-275 g at the time of arrival; Harlan Labs, Madison, WI) were housed in temperature (23±3°C) and light (12:12 light:dark; lights on at 0700 hr) controlled rooms with standard rodent chow and water available *ad libitum*. The route of drug delivery for all experiments was intrathecal (sub-dural, peri-spinal) and took 2-3 minutes to complete. An acute catheter application method under brief isoflurane anesthesia (3.0% volume in oxygen) was employed, as described previously³⁰, to inject BDNF or PEG-BDNF at the level of the lumbosacral enlargement.

In vivo half-life assessment

At pre-determined time points after intrathecal injections (30, 90, 120 and 240 minutes), lumbosacral (lumbar) CSF samples were collected under isoflurane anesthesia³¹, after which point the rats were immediately euthanized. CSF was collected from six animals at each time point. CSF samples were immediately transferred to dry ice and samples were subsequently stored at -80°C for further analysis. An ELISA kit was used for the quantitative detection of BDNF according to manufacturer protocols (Promega).

In vivo bio-distribution assessment

Transcardial perfusions were performed as previously described^{32,33} with 0.9% saline (5 minutes) followed by chilled, fresh 2% paraformaldehyde in 0.1% PBS (5 minutes) on two rats for each condition at the 240 minute time point. Cord sections were collected and post-fixed in 4% paraformaldehyde overnight at 4°C and then cryoprotected in a 30% sucrose solution. Sections were frozen while embedded in OCT Compound (Tissue-Tek), cryostat sectioned at 10 microns and thaw mounted onto Superfrost Plus Slides (Fisher).

For immunohistochemical analysis, the slide mounted sections were washed in PBS and incubated in a non-specific protein block solution for 3 hours. A polyclonal antibody for BDNF (Chemicon Ab1779) was diluted 1:1000 in blocking solution and applied overnight at 4°C. Slides were rinsed briefly in blocking solution and an AlexaFluor546 secondary antibody (Molecular Probes) was diluted 1:200 in blocking solution and applied for four hours. For the detection of cell nuclei the slides were incubated with 1:1000 diluted DAPI (Molecular Probes) for 10 minutes, washed and incubated in PBS overnight. Cover-slips were then mounted onto the slides by applying 12-15 µl of Fluoromount-G (Fisher) to each tissue region. The sections were examined with confocal microscopy using a Zeiss Pascal LSM microscope with a 40x Plan NeoFluor (1.3) oil immersion objective.

Line intensity profiles on acquired images were collected with NIH ImageJ software. Confocal images for BDNF pAb staining of spinal cord cross sections were converted to black and white and the pixel length was calibrated to cord length in µm. The plot profile tool was then used to measure the fluorescence intensity as a function of distance from the edge of the cord. Fluorescence intensity values were averaged from eight different sections from two different rats at each condition for a total of 80 different profiles per condition. Data were normalized against the fluorescence intensity at the edge of the cord for each profile. The 50% penetration distance was reported as the point where the average fluorescence intensity was 50% of the average fluorescence intensity at the edge of the cord.

Results

Mass spec characterization of PEG-BDNF conjugates

MALDI-TOF mass spectrometry was used to determine the molecular weight of each species present in a given conjugate mixture. Here we provide representative mass spec profiles for the PEG used for conjugation, BDNF and a conjugate mixture produced by reacting a 60-fold molar excess of PEG with BDNF in the presence of 125-fold molar excess of reducing agent. Unreacted polymer (5 kDa mPEG-ButyrALD) had a molecular weight of 5.81 kDa which is consistent with manufacturer claims (Fig. 2a). The molecular weight of monomeric BDNF was 13.6 kDa (Fig. 2b), which is also consistent with manufacturer claims. The peak at 6.82 kDa was the doubly-charged m/z species of the BDNF monomer.

Three additional peaks with molecular weights of 20.0 kDa, 26.2 kDa and 32.2 kDa were observed in the conjugate mixture that were not seen in the profiles for the unreacted polymer and BDNF (Fig. 2c). The molecular weight values obtained from mass spectrometry are consistent with the attachment of one (primary conjugate), two (secondary conjugate), or three (tertiary conjugate) 5.81 kDa PEG molecules to BDNF. The peak at

10.0 kDa was the doubly-charged m/z species of the primary conjugate. While SDS-PAGE analysis cannot provide quantitative molecular weight information regarding PEGylated proteins, similar qualitative results were obtained. In addition to unreacted BDNF, three conjugate bands of increasing molecular weight were observed in SDS-PAGE corresponding to primary, secondary, and tertiary conjugate (Fig. 3a, 60X PEG to BDNF molar excess).

Production of mixtures enriched in primary and secondary conjugates

In order to generate PEG-BDNF mixtures with varying amounts of primary, secondary, and higher order conjugates, the influence of reaction conditions on the amount of and type of conjugate generated was explored. Fixing the reducing agent concentration at 125-fold molar excess and increasing the amount of PEG present in the reaction buffer from one-fold molar excess to ten-fold increased the amount of primary and secondary conjugate formed (Fig. 3a). Mixtures composed of primary and secondary conjugate species were also produced when the reducing agent concentration was decreased to 10-60-fold excess and PEG levels were held at 60-fold excess (Fig. 3b).

Mixtures containing primary, secondary, and tertiary conjugate were formed at high reducing agent concentration (125-fold excess) in the presence of high amounts of PEG (15-fold excess to 60-fold excess) (Fig. 3a). Specifically, the amount of primary conjugate formed decreased and the amount of secondary and tertiary conjugate formed increased as PEG excess increased from 15-fold to 60-fold (Fig. 3a). In the presence of high levels of PEG (60-fold excess), increasing the reducing agent concentration to 600-fold excess resulted in the production of primary, secondary, tertiary, and higher-order conjugate species (Fig. 3c). Using mPEG-SPA instead of mPEG-ButyrALD (i.e. NHS ester chemistry instead of aldehyde chemistry) resultant mixtures were also highly enriched in tertiary and higher order conjugate products at 15-fold and 60-fold PEG to BDNF molar excess values (data not shown).

Based on these findings, we identified the following approaches to creating mixtures with different fractions of primary, secondary, tertiary or higher order conjugate species. When 125-fold excess reducing agent is present in solution, conjugate mixtures containing an abundance of primary conjugate and free BDNF can be formed in the presence of one-fold or two-fold excess PEG; a 10-fold excess of PEG results in the production of a mixture of primary, secondary, and tertiary conjugates. Mixtures enriched in primary and secondary conjugate are produced in the presence of lower levels of reducing agent (60-fold excess) and 10-fold or 60-fold excess PEG. Mixtures enriched in primary, secondary, tertiary and higher order conjugate species are prepared in the presence of higher levels of reducing agent (600-fold excess) and 60-fold excess PEG, or by PEGylating BDNF using NHS-ester chemistry.

A comparison of coomassie staining and immunoblotting to detect PEGylated BDNF conjugate mixtures prepared with aldehyde chemistry was also conducted (Fig. 3c). Primary and secondary conjugates were detected by antibodies for BDNF. However, tertiary and greater than tertiary conjugate species were not detected by the antibody for BDNF.

Biological activity assessment

A neurite extension assay with the PC12-trkB cell line was conducted in order to assess the *in vitro* biological activity of various conjugate mixtures. The PC12-trkB cell line, which stably expresses the trkB receptor, extends neurites from the cell body in the presence of functional BDNF³⁴. The mean percent of neurite extension was assessed by counting the fraction of cells extending neurites two times greater than the length of the cell body. As the concentration of BDNF increased linearly from 0.1 to 0.5 ng/ml, the percentage of cells extending neurites increased linearly (Fig. 4a).

The presence of non-covalently attached PEG (no reducing agent) did not alter the *in vitro* biological activity of BDNF (Fig. 4b, 60X PEG). When BDNF was incubated with reducing agent in the absence of PEG, slight decreases in biological activity occurred with increasing amounts of reducing agent (Fig. 4b 60X RA – 240X RA). At reducing agent to BDNF molar excess values of 360-fold or greater, minor biological activity reductions became more significant ($p < 0.05$) reaching an ultimate reduction of 1.2-fold ($p < 0.05$) with a 600-fold molar excess of reducing agent to BDNF (Fig. 4b 360X – 600X).

Conjugate mixtures enriched with different fractions of free BDNF, primary, secondary, tertiary, and higher-order species were tested for biological activity. Mixtures enriched in primary conjugate exhibited slightly reduced *in vitro* biological activities relative to unmodified BDNF, however, the measured reductions were not statistically significant (Fig. 5a: 1/125, 2/125). Full bioactivity was preserved in mixtures composed of primary and secondary conjugates (Fig. 5b: 60/10, 60/60). A 1.5-fold ($p < 0.001$) reduction in activity was observed in the mixture composed of primary, secondary, and tertiary conjugate (Fig. 5c: 60/125). The reduction in activity is likely due to the presence of higher levels of tertiary conjugate. An 8.4-fold ($p < 0.001$) reduction in biological activity was also observed in mixtures containing large amounts of tertiary and higher order conjugates (Fig. 5c: 60/600).

The *in vitro* biological activity was also assessed for (PEG-SPA) conjugate mixtures produced by NHS ester reaction chemistry (Fig. 5d) as this reaction was able to generate extensive PEGylation without the need for a reducing agent. A PEG to BDNF molar excess of 15-fold resulted in the production of a mixture of secondary, tertiary, and higher order conjugates with a 3.6-fold ($p < 0.001$) reduction of *in vitro* biological activity (Fig. 5d: 15/0 (SPA)). Increasing the PEG to BDNF molar excess to 60-fold generated a mixture dominated by higher order conjugates (>84%) with a 10.8-fold ($p < 0.001$) biological activity reduction (Fig. 5d: 60/0 (SPA)).

In vivo CSF Half-life Analysis

In order to determine whether an improvement in CSF half-life is observed when a PEGBDNF mixture enriched in primary and secondary conjugate products is administered intrathecally, the conjugate mixture utilizing a 60-fold molar excess of PEG and a 60-fold molar excess of reducing agent was administered *in vivo*. This conjugate mixture was selected over the mixture with the 10-fold molar excess of reducing agent and the same molar excess of PEG due to the fact that it contained a lower amount of free BDNF even though both mixtures exhibited a fully preserved *in vitro* biological activity.

The concentration of PEG-BDNF in the CSF after intrathecal administration was compared to unmodified BDNF over time. ELISA results demonstrated that the PEG-BDNF mixture was detected with the same avidity as the BDNF stock (data not shown). At predetermined time points after intrathecal injection, CSF samples collected at the lumbar region demonstrated that PEG-BDNF was much more abundant than BDNF over the entire testing interval. The concentration of PEG-BDNF relative to BDNF was six-fold ($p<0.005$) higher after 30 minutes, six- to seven-fold higher ($p<0.05$) at 90 and 120 minutes and 12.6-fold higher ($p<0.005$) at 240 minutes (Fig. 6). From this data, the half-life of PEG-BDNF in the CSF was calculated to be 167 minutes while the half-life of BDNF in the CSF was 62.7 minutes, values which suggest a 2.6-fold improvement in half-life for the fully active PEG-BDNF conjugate mixture.

Tissue Penetration Profiling

The fully bioactive PEG-BDNF conjugate mixture exhibited a prolonged bioavailability in the CSF. The prolonged stability of the protein conjugate mixture in the CSF may be due to the increased size of the protein, sterically hindering proteases from degrading BDNF. While improvements in CSF availability of the protein were achieved, the ability of the conjugate mixture to penetrate into parenchymal tissue to reach cellular targets must also be assessed. Molecules that are larger in molecular weight tend to diffuse more slowly in tissue environments. Thus, it is possible that the availability of the conjugated protein to cellular targets in parenchymal tissue would go down, due to a decrease in the rate at which the protein-conjugates diffuse through tissue. For this reason, the extent to which the conjugate mixture was capable of penetrating into the surrounding parenchymal tissue following intrathecal delivery was evaluated.

Lumbar region spinal cord tissues were collected four hours after intrathecal injections of BDNF and PEG-BDNF. Sections were fixed, immuno-stained for BDNF, and imaged by confocal microscopy. BDNF was primarily detected on the periphery of spinal cord tissue (Fig. 7a). PEG-BDNF was detected at high concentrations in the cord periphery, but was also detected at elevated levels within the tissue (Fig. 7b). Control tissue (no injection) did not exhibit background staining for human BDNF (data not shown). Line profiles of the relative fluorescence intensity vs. tissue depth confirmed that the PEG-BDNF conjugate mixture penetrated deeper into the spinal cord tissue than BDNF (Fig. 7c). Comparisons of the fluorescence intensity values indicate that the PEG-BDNF conjugate mixture declined to 50% of its initial value at a depth of 34.57 μm compared to 8.02 μm for the BDNF only injections. Consistent with the improvement in CSF protein availability, the fully bioactive PEG-BDNF conjugate mixture also has improved availability in parenchymal tissue.

Discussion

Many PEGylation strategies have focused on the preservation of *in vitro* biological activity as an important criterion for therapeutic development^{10,21-23,28,35,36}. Site-specific PEGylation strategies to minimize disruption of the binding site are often the most advantageous approach for biological activity preservation^{12,22,28,36-38}. The functional sites for BDNF are the trkB and p75 receptor binding regions. The aldehyde reaction chemistry

used in this work preferentially targets the N-terminus of a protein and the N-terminus of BDNF is not involved in p75 and trkB receptor binding³⁹. Five out of the eleven lysine residues (potentially reactive amines) on BDNF are within the trkB binding region, four of which are also located within the p75 binding region³⁹. Preferential N-terminal PEGylation increases the potential for bioactivity preservation as it decreases the potential for attachment with these lysine residues in a functional region. The full preservation of *in vitro* biological activity with the attachment of one PEG to BDNF for mixtures enriched with PEG-BDNF primary conjugates is likely due to the attachment of PEG at the N-terminus by nature of the reaction chemistry.

While aldehyde chemistry is designed to preferentially attach a PEG molecule to the N-terminus, increasing the molar excess of PEG creates additional covalent linkages between PEG molecules and surface lysine groups which subsequently decreases the yield of mono PEGylated species^{12,29,40,41}. In other words, even though the covalent attachment of PEG to a protein with aldehyde chemistry is preferential for the N-terminus, other amine containing groups on the protein are still reactive to PEG due to their accessibility and the presence of unreacted PEG. In a PEGylated mixture of interferon- β peptide mapping has demonstrated that primary conjugates consist almost entirely (>90%) of PEG attached to the N-terminal peptide and that secondary covalent linkages preferentially occur between PEG and a single accessible lysine group⁴². Similar results demonstrating preferential N-terminal attachment for the primary conjugate species and subsequent attachment to accessible lysine groups for the multi-PEGylated species using site-directed aldehyde chemistry have also been found with EGF¹², tumor necrosis factor⁴¹ and GM-CSF¹⁶. Therefore, even though the preferential site of PEG attachment is at the N-terminus, the result that increasing the molar excess of PEG and/or reducing agent increased the formation of multi-PEGylated species is consistent with previous findings.

In general, the clearance rate of a protein is reduced as the number of PEG molecules attached to a protein is increased^{23,24,43,44}. However, as the functional sites for BDNF binding to neurotrophins contain lysine residues, increasing the number of PEG molecules attached to lysine groups on the protein will increase the potential for binding disruptions. For this reason, a goal of this study was to identify the maximum number of PEG molecules that could be attached to BDNF without compromising its biological activity. The *in vitro* biological activity was fully preserved in the mixtures enriched with primary and secondary conjugates. This demonstrates that conjugates in a mixture with large amounts of BDNF bound to one or two PEG molecules were functional, or in other words that the preferential attachment site for a second PEG molecule is not disruptive to biological activity, suggesting that the second PEG molecule is not attached to a surface lysine group within the trkB or p75 receptor binding regions of BDNF.

Increasing the fraction of tertiary and higher order conjugates in a mixture, on the other hand, reduced the *in vitro* biological activity as PEG-BDNF conjugate mixtures with increasing levels of higher order conjugates exhibited decreased biological activities. This indicates that attaching several PEG molecules to BDNF reduces access to binding sites on the molecule. The polyclonal antibody used in this work neutralizes the bioactivity of BDNF applied to PC12-trkB cells in culture (data not shown) and manufacturer specifications for

the antibody indicate that it neutralizes the bioactivity of BDNF but not other neurotrophins. Immunoblotting data using this antibody for BDNF demonstrated that it cannot detect tertiary and higher order conjugates in the mixture with high avidity (Fig. 3c), indicating that antibody binding was disrupted for these conjugate species. As the trkB receptor is more specific for BDNF than the other neurotrophins³⁹ this leads to the conclusion that tertiary and higher order PEG attachments likely occur on lysine residues within or near to the trkB receptor binding regions of BDNF.

Elevated concentrations of PEG-BDNF in the CSF were recognized over the course of 6 hours, long enough to match the CSF turnover rate in humans⁴⁵, even though the entire CSF volume turns over every 2-4 hours for a rat. This turnover is essentially one-way, where CSF from the subarachnoid space is cleared to the bloodstream or lymph nodes⁴⁵⁻⁴⁸. The increased ability of PEG-BDNF to diffuse in and out of surface tissue to avoid clearance, along with the shielding effects of PEGylation against proteolytic and enzymatic degradation products in the CSF and surface tissue, are likely responsible for its increased persistence in the CSF over time. In the bloodstream, PEGylation can shield a protein from enzymatic degradation and antigenic determinants of the immune system^{35,49}. These agents are less abundant in the CSF than in the bloodstream, but there is increasing evidence for the presence of serine proteases and antigenic determinants in the CSF^{31,50}, in addition to their presence in spinal cord tissue. PEGylation of BDNF has been shown to reduce its rate of clearance from the bloodstream by nearly 10-fold²¹. The 2.67-fold improvement in clearance from the CSF was more moderate than results in the bloodstream, but is consistent with the high turnover rate of products from the CSF and reduced protease levels and components of the immune system in the CSF when compared to the bloodstream.

To improve efficacy following intrathecal administration, the PEGylated protein must have improved stability in the CSF and its penetration into the spinal cord tissue must be improved. PEG-BDNF conjugate species in these studies exhibited enhanced penetration into spinal cord tissue (Fig. 7c). Even though larger molecules typically exhibit a reduced ability to diffuse through tissue, PEGylation creates a hydration layer around a protein which increases its solubility^{35,51}, reduces its non-specific electrostatic interactions¹, and shields it from receptor mediated uptake by surface tissues thereby limiting its availability to the interior tissue^{2,26}. Therefore even though PEG-BDNF conjugate species are larger in size than BDNF, PEGylated species would be expected to exhibit enhanced diffusion into spinal cord tissue. Prior work has shown that PEG-BDNF exhibits enhanced diffusion in *ex vivo* brain tissue slices¹ and *in vivo* penetration into the spinal column and forebrain after prolonged exposure to continuous intrathecal infusions². Consistent with and improving upon these findings, we have shown that improved diffusion of PEG-BDNF into spinal cord tissue *in vivo* also occurred after a single intrathecal injection.

Conclusions

Ongoing work continues to validate the merits of PEGylation for improving the overall efficacy of therapeutic proteins. Directed and controlled PEGylation is a promising approach enabling a high preservation of *in vitro* biological activity with an improved *in vivo* pharmacokinetic profile after intrathecal delivery. PEGylation of BDNF using aldehyde

chemistry for control of primary and secondary conjugate formation preserved the *in vitro* biological activity of the mixture while improving its penetration into spinal cord tissue and half-life in the CSF. The half-life was improved following intrathecal administration at a duration that is large enough in magnitude to be effective in humans. The approach herein used for the PEGylation of BDNF could also be extended to other therapeutic proteins that must be delivered intrathecally to reduce dosage requirements and prolong the therapeutic efficacy of treatments for a wide range of central nervous system disorders.

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References

1. Stroh M, Zipfel WR, Williams RM, Ma SC, Webb WW, Saltzman WM. Multiphoton microscopy guides neurotrophin modification with poly(ethylene glycol) to enhance interstitial diffusion. *Nature Materials*. 2004; 3(7):489–494.
2. Ankeny DP, McTigue DM, Guan Z, Yan Q, Kinstler O, Stokes BT, Jakeman LB. Pegylated brain-derived neurotrophic factor shows improved distribution into the spinal cord and stimulates locomotor activity and morphological changes after injury. *Experimental Neurology*. 2001; 170(1): 85–100. [PubMed: 11421586]
3. Kishino A, Katayama N, Ishige Y, Yamamoto Y, Ogo H, Tatsuno T, Mine T, Noguchi H, Nakayama C. Analysis of effects and pharmacokinetics of subcutaneously administered BDNF. *Neuroreport*. 2001; 12(5):1067–1072. [PubMed: 11303747]
4. Encinas M, Iglesias M, Llecha N, Comella JX. Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. *Journal Of Neurochemistry*. 1999; 73(4):1409–1421. [PubMed: 10501184]
5. Miki K, Fukuoka T, Tokunaga A, Kondo E, Dai Y, Noguchi K. Differential effect of brain-derived neurotrophic factor on high-threshold mechanosensitivity in a rat neuropathic pain model. *Neuroscience Letters*. 2000; 278(1-2):85–88. [PubMed: 10643807]
6. Eaton MJ, Blits B, Ruitenberg MJ, Verhaagen J, Oudega M. Amelioration of chronic neuropathic pain after partial nerve injury by adeno-associated viral (AAV) vector-mediated over-expression of BDNF in the rat spinal cord. *Gene Therapy*. 2002; 9(20):1387–1395. [PubMed: 12365004]
7. Bergman I, Burckart GJ, Pohl CR, Venkataramanan R, Barmada MA, Griffin JA, Cheung NKV. Pharmacokinetics of IgG and IgM anti-ganglioside antibodies in rats and monkeys after intrathecal administration. *Journal Of Pharmacology And Experimental Therapeutics*. 1998; 284(1):111–115. [PubMed: 9435168]
8. Veronese FM, Caliceti P, Schiavon O, Sergi M. Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Advanced Drug Delivery Reviews*. 2002; 54(4):587–606. [PubMed: 12052716]
9. Dang W, Colvin MO, Brem H, Saltzman WM. Covalent coupling of methotrexate to dextran enhances the penetration of cytotoxicity into a tissue-like matrix. *Cancer Research*. 1994; 54:1729–1735. [PubMed: 7511049]
10. Francis GE, Fisher D, Delgado C, Malik F, Gardiner A, Neale D. PEGylation of cytokines and other therapeutic proteins and peptides: the importance of biological optimisation of coupling techniques. *International Journal of Hematology*. 1998; 68:1–18. [PubMed: 9713164]
11. Belcheva N, Woodrow-Mumford K, Mahoney MJ, Saltzman WM. Synthesis and biological activity of polyethylene glycol-mouse nerve growth factor conjugate. *Bioconjugate Chemistry*. 1999; 10(6):932–937. [PubMed: 10563761]

12. Lee H, Jang IH, Ryu SH, Park TG. N-terminal site-specific mono-PEGylation of epidermal growth factor. *Pharmaceutical Research*. 2003; 20(5):818–825. [PubMed: 12751640]
13. Krinner EM, Hepp J, Hoffmann P, Bruckmaier S, Petersen L, Petsch S, Parr L, Schuster I, Mangold S, Lorenczewski G. A highly stable polyethylene glycol-conjugated human single-chain antibody neutralizing granulocyte-macrophage colony stimulating factor at low nanomolar concentration. *Protein Engineering Design & Selection*. 2006; 19(10):461–470. others.
14. Yun Q, Xing WC, Mal GG, Su ZG. Preparation and characterization of mono-PEGylated consensus interferon by a novel polyethylene glycol derivative. *Journal Of Chemical Technology And Biotechnology*. 2006; 81(5):776–781.
15. Ramon J, Saez V, Baez R, Aldana R, Hardy E. PEGylated interferon-alpha 2b: A branched 40K polyethylene glycol derivative. *Pharmaceutical Research*. 2005; 22(8):1374–1386. [PubMed: 16078148]
16. Kinstler OB, Brems DN, Lauren SL, Paige AG, Hamburger JB, Treuheit MJ. Characterization and stability of N-terminally PEGylated rhG-CSF. *Pharmaceutical Research*. 1996; 13(7):996–1002. [PubMed: 8842035]
17. Tsutsami Y, Tsunoda S-I, Kamada H, Kihira T, Kaneda Y, Ohsugi Y, Mayumi T. PEGylation of Interleukin-6 Effectively Increases Its Thrombopoietic Potency. *Thrombosis and Haemostasis*. 1997; 77(1):168–173. [PubMed: 9031469]
18. Pardridge WM, Wu D, Sakane T. Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. *Pharmaceutical Research*. 1998; 15(4):576–582. [PubMed: 9587954]
19. Katre NV, Knauf MJ, Laird WL. Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. USA*. 1987; 84:1487–1491. [PubMed: 3494243]
20. Hudecz F, Clegg JA, Kajtar J, Embleton MJ, Pimm MV, Szekerke M, Baldwin RW. Influence of carrier on biodistribution and in vitro cytotoxicity of methotrexate-branched polypeptide conjugates. *Bioconjugate Chemistry*. 1993; 4:25–33. [PubMed: 8431509]
21. Sakane T, Pardridge WM. Carboxyl-directed pegylation of brain-derived neurotrophic factor markedly reduces systemic clearance with minimal loss of biologic activity. *Pharmaceutical Research*. 1997; 14(8):1085–1091. [PubMed: 9279893]
22. Leong SR, DeForge L, Presta L, Gonzalez T, Fan A, Reichert M, Chuntharapai A, Kim KJ, Tumas DB, Lee WP. Adapting pharmacokinetic properties of a humanized anti-interleukin-8 antibody for therapeutic applications using site-specific pegylation. *Cytokine*. 2001; 16(3):106–119. others. [PubMed: 11741351]
23. Koumenis IL, Shahrokh Z, Leong S, Hsei V, Deforge L, Zapata G. Modulating pharmacokinetics of an anti-interleukin-8 F(ab')₂ by amine-specific PEGylation with preserved bioactivity. *International Journal Of Pharmaceutics*. 2000; 198(1):83–95. [PubMed: 10722953]
24. Clark R, Olson K, Fuh G, Marian M, Mortensen D, Teshima F, Chang S, Chu H, Mukku V, CanovaDavis E. Long-acting growth hormones produced by conjugation with polyethylene glycol. *Journal Of Biological Chemistry*. 1996; 271(36):21969–21977. others. [PubMed: 8703002]
25. Bailon P, Berthold W. Polyethylene glycol-conjugated pharmaceutical proteins. *Pharmaceutical Science & Technology Today*. 1998; 1(8):352–356.
26. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews*. 2002; 54(4):459–476. [PubMed: 12052709]
27. Kinstler O, Molineux G, Treuheit M, Ladd D, Gegg C. Mono-N-terminal poly(ethylene glycol)-protein conjugates. *Advanced Drug Delivery Reviews*. 2002; 54(4):477–485. [PubMed: 12052710]
28. Rosendahl MS, Doherty DH, Smith DJ, Carlson SJ, Chlipala EA, Cox GN. A long-acting, highly potent interferon alpha-2 conjugate created using site-specific PEGylation. *Bioconjugate Chemistry*. 2005; 16(1):200–207. [PubMed: 15656592]
29. Na DH, Lee KC, DeLuca PP. PEGylation of octreotide: II. Effect of N-terminal mono-PEGylation on biological activity and pharmacokinetics. *Pharmaceutical Research*. 2005; 22(5):743–749. [PubMed: 15906169]

30. Milligan ED, Langer SJ, Sloane EM, He L, Wieseler-Frank J, O'Connor K, Martin D, Forsayeth JR, Maier SF, Johnson K. Controlling pathological pain by adenovirally driven spinal production of the anti-inflammatory cytokine, interleukin-10. *European Journal Of Neuroscience*. 2005; 21(8):2136–2148. others. [PubMed: 15869510]
31. Milligan ED, O'Connor KA, Nguyen K, Armstrong CB, Twining C, Gaykema RPA, Holguin A, Martin D, Maier SF, Watkins LR. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *Journal Of Neuroscience*. 2001; 21(8):2808–2819. [PubMed: 11306633]
32. Mannes A, Caudle R, O'Connell B, Iadarola M. Adenoviral gene transfer to spinal cord neurons: intrathecal vs. intraparenchymal administration. *Brain Research*. 1998; 793(1-2):1–6. [PubMed: 9630472]
33. Milligan ED, Sloane EM, Langer SJ, Cruz PE, Chacur M, Spataro L, Wieseler-Frank J, Hammack SE, Maier SF, Flotte TR. Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. *Molecular Pain*. 2005; 1(9):1–9. others. [PubMed: 15813987]
34. Greene LA, Aletta JM, Rukenstein A, Green SH. PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Methods in Enzymology*. 1987; 147:207–216. [PubMed: 3670084]
35. Molineux G. Pegylation: Engineering Improved Biopharmaceuticals for Oncology. *Pharmacotherapy*. 2003; 23(8 Pt 2):3S–8S. [PubMed: 12921216]
36. Gaertner H, Offord R. Site-specific attachment of functionalized poly(ethylene glycol) to the amino terminus of proteins. *Bioconjugate Chemistry*. 1996; 7(1):38–44. [PubMed: 8741989]
37. Lee H, Park TG. Preparation and characterization of mono-PEGylated epidermal growth factor: Evaluation of in vitro biologic activity. *Pharmaceutical Research*. 2002; 19(6):845–851. [PubMed: 12134956]
38. Kubetzko S, Sarkar CA, Pluckthun A. Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Molecular Pharmacology*. 2005; 68(5):1439–1454. [PubMed: 16099846]
39. Robinson RC, Radziejewski C, Stuart DI, Jones EY. Structure Of The Brain-Derived Neurotrophic Factor Neurotrophin 3 Heterodimer. *Biochemistry*. 1995; 34(13):4139–4146. [PubMed: 7703225]
40. Cindric M, Cepo T, Galic N, Bukvic-Krajacic M, Tomczyk N, Vissers JPC, Bindila L, Peter-Katalinic J. Structural characterization of PEGylated rHuG-CSF and location of PEG attachment sites. *Journal Of Pharmaceutical And Biomedical Analysis*. 2007; 44(2):388–395. [PubMed: 17448619]
41. Edwards CK, Martin SW, Seely J, Kinstler O, Buckel S, Bendele AM, Cosenza ME, Feige U, Kohno T. Design of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) for chronic inflammatory diseases. *Advanced Drug Delivery Reviews*. 2003; 55(10):1315–1336. [PubMed: 14499710]
42. Arduini RM, Li ZF, Rapoza A, Gronke R, Hess DM, Wen DY, Miatkowski K, Coots C, Kaffashan A, Viseux N. Expression, purification, and characterization of rat interferon-beta, and preparation of an N-terminally PEGylated form with improved pharmacokinetic parameters. *Protein Expression And Purification*. 2004; 34(2):229–242. others. [PubMed: 15003256]
43. Baker DE. Pegylated Interferons. *Reviews in Gastroenterological Disorders*. 2001; 1(2):87–99. [PubMed: 12120178]
44. Eliason JF. Pegylated cytokines: potential application in immunotherapy of cancer. *BioDrugs*. 2001; 15(11):705–711. [PubMed: 11707145]
45. Kandel, ER.; Schwartz, JH.; Jessell, TM. *Principles of Neural Science*. McGraw-Hill; 2000.
46. Weller RO, Kida S, Zhang ET. Pathways Of Fluid Drainage From The Brain - Morphological Aspects And Immunological Significance In Rat And Man. *Brain Pathology*. 1992; 2(4):277–284. [PubMed: 1341963]
47. Johnston M. The importance of lymphatics in cerebrospinal fluid transport. *Lymphatic Research and Biology*. 2003; 1(1):41–45. [PubMed: 15624320]
48. Abbott NJ. Evidence for bulk flow of brain interstitial fluid: significance for physiology and pathology. *Neurochemistry International*. 2004; 45(4):545–552. [PubMed: 15186921]

49. Greenwald RB, Yang K, Zhao H, Conover CD, Lee S, Filpula D. Controlled release of proteins from their poly(ethylene glycol) conjugates: drug delivery systems employing 1,6-elimination. *Bioconjugate Chemistry*. 2003; 14(2):395–403. [PubMed: 12643750]
50. Scarisbrick IA, Towner MD, Isackson PJ. Nervous system-specific expression of a novel serine protease: Regulation in the adult rat spinal cord by excitotoxic injury. *Journal Of Neuroscience*. 1997; 17(21):8156–8168. [PubMed: 9334391]
51. Dhalluin C, Ross A, Leuthold LA, Foser S, Gsell B, Muller F, Senn H. Structural and biophysical characterization of the 40 kDa PEG-interferon-alpha(2a) and its individual positional isomers. *Bioconjugate Chemistry*. 2005; 16(3):504–517. [PubMed: 15898716]

Figure 1. Reaction diagrams of (a) conjugation with mPEG-ButyrALD and (b) conjugation with mPEG-SPA.

Figure 2. MALDI-TOF mass spectrometry of (a) PEG species used for conjugation, (b) BDNF and (c) PEG-BDNF conjugate mixture prepared with a 60-fold excess of PEG to BDNF and a 125-fold excess of reducing agent to BDNF.

Figure 3.

SDS-PAGE analysis (10% Gels) of conjugate mixtures. (a) PEG-BDNF mixtures prepared with a 125-fold excess of reducing agent (RA) to BDNF with varied molar excesses of PEG to BDNF detected by coomassie staining. (b) PEG-BDNF mixtures prepared with a 60-fold excess of PEG to BDNF and either a 10-fold or a 60-fold molar excess of RA to BDNF detected by coomassie staining. (c) PEG-BDNF mixtures with a 60-fold molar excess of PEG to BDNF and a 600-fold molar excess of RA to BDNF detected by coomassie staining and immunoblotting.

Figure 4.

PC12-trkB neurite extension results. (a) Application of BDNF at concentrations in the range of 0 to 0.5 ng/ml and (b) Application of 0.5 ng/ml of BDNF treated with PEG only or reducing agent (RA) only at the indicated molar excess values. * indicates p value < 0.05 relative to BDNF only condition (two-tailed t-test, error bars are standard error of the mean).

Figure 5.

PC12-trkB neurite extension results after the application of BDNF and PEG-BDNF conjugate mixtures at a concentration of 0.5 mg/ml (total BDNF for all species). ‘--’ represents the expected degree of neurite extension from residual free BDNF levels in each mixture (free BDNF level obtained from band density analysis of coomassie stained gels, and estimated degree of neurite extension obtained from the dose-response relationship in Fig. 4a). (a) PEG-BDNF mixtures enriched in primary conjugate species. (b) PEG-BDNF mixtures containing primary and secondary conjugate species. (c) PEG-BDNF mixtures containing tertiary and higher order conjugate species. (d) PEG-BDNF mixtures prepared with mPEG-SPA instead of mPEGButyrALD. m/n indicates m-fold excess PEG to BDNF and n-fold excess reducing agent to BDNF respectively. * indicates p value < 0.001 relative to control BDNF (two-tailed t-test, error bars are standard error of the mean).

Figure 6.

ELISA detection of BDNF in lumbar CSF samples after intrathecal injections of BDNF or PEG-BDNF. * and ** indicate p values < 0.05 and <0.005, respectively, for time-matched PEG-BDNF compared to BDNF (unequal variance two-tailed t-test, error bars are standard error of the mean).

Figure 7. Confocal imaging along the periphery of BDNF immuno-stained lumbar spinal cord tissue sections. (a) Spinal cord cross-section 4 hours after intrathecally injected BDNF. (b) Spinal cord cross-section 4 hours after intrathecally injected PEG-BDNF. (c) ImageJ analysis of fluorescence intensity values relative to distance from the spinal cord cross-section periphery.