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Nano-particle Delivery of Brain Derived Neurotrophic Factor after Focal Cerebral Ischemia Reduces Tissue Injury and Enhances Behavioral Recovery

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Abstract

Background—Low levels of brain-derived neurotrophic factor (BDNF) are linked to delayed neurological recovery, depression, and cognitive impairment following stroke. Supplementation with BDNF reverses these effects. Unfortunately, systemically administered BDNF in its native form has minimal therapeutic value due to its poor blood brain barrier permeability and short serum half-life. In this study, a novel nano-particle polyion complex formulation of BDNF (nano-BDNF) was administered to mice after experimental ischemic stroke.

Methods—Male C57BL/6J (8–10 weeks) mice were randomly assigned to receive nano-BDNF, native-BDNF, or saline treatment after being subjected to 60 minutes of reversible middle cerebral artery occlusion (MCAo). Mice received the first dose at 3 (early treatment), 6 (intermediate treatment), or 12 hours (delayed treatment) following stroke onset; a second dose was given in all cohorts at 24 hours after stroke onset. Post-stroke outcome was evaluated by behavioral, histological, and molecular analysis for 15 days after stroke.

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Results—Early and intermediate nano-BDNF treatment led to a significant reduction in cerebral tissue loss. Delayed treatment led to improved memory/cognition, reduced post-stroke depressive phenotypes, and maintained myelin basic protein and brain BDNF levels, but had no effect on tissue atrophy.

Conclusions—The results indicate that administration of a novel nano-particle formulation of BDNF leads to both neuroprotective and neuro-restorative effects after stroke.

Keywords

Stroke; Neuroprotection; Neuro-restorative; BDNF; Depressive behavior

INTRODUCTION

Stroke is the fifth leading cause of death in the United States and is a leading cause of adult disability (Mozaffarian et al., 2016). The long lasting impairments seen after stroke diminish independence and reduce quality of life in stroke survivors (Synhaeve et al., 2015). In addition to physical disability and cognitive impairments, post stroke depression (PSD) is also a frequent and important neuropsychiatric consequence of stroke (Lokk and Delbari, 2010). About 40% of stroke survivors suffer from PSD (Lenzi et al., 2008). PSD develops soon after stroke, and can persist for years and impair neurological recovery after stroke (Zavoreo et al., 2009).

Given that stroke significantly impairs quality of life, it is imperative to find treatments that improve functional recovery after stroke onset. Brain derived neurotrophic factor (BDNF) induces neuronal plasticity and plays an important role in post stroke rehabilitation and recovery (Mang et al., 2013). Moreover, exogenous supplementation or overexpression of BDNF can reduce infarct volume, improve neurological outcome, enhance sensorimotor function and mediates both oligodendrogenesis and remyelination after white matter injury in experimental stroke models (Lee et al., 2010; Ramos-Cejudo et al., 2015; Schabitz et al., 2007; Yasuhara et al., 2008).

Several laboratories, including ours, have shown that neuronal expression of BDNF increases acutely after stroke as a pro-survival response and that BDNF levels correlate with the degree of functional survival (Bejot et al., 2011; O'Keefe et al., 2014). Decreased or low BDNF levels have been associated with depression and cognitive impairment (Zhang et al., 2012). Given the fact that BDNF has both neuroprotective and antidepressant/neuro-restorative effects (Allen et al., 2015), BDNF may be a useful treatment strategy for acute stroke and post-stroke functional deficits. Despite the known benefits of BDNF, its usefulness in treating stroke, depression, and other neurological disorders is limited due to its poor accumulation by brain and short serum half-life (Pan et al., 1998). Although stroke disrupts the BBB and increases vascular permeability, this does not occur until several hours after stroke onset (Fluri et al., 2015; Hong et al., 2015). Furthermore, the BBB leakages that occur with stroke are often not of such a magnitude to allow large proteins, such as BDNF, to achieve substantial pharmacological levels within the brain (Sullivan et al., 2011).

In order to have therapeutic value, these obstacles must be overcome either by conjugating BDNF to a BBB drug delivery system or by optimizing the pharmacokinetics of BDNF to increase plasma half-life. In this study, we utilized a polymeric nano-formulation of BDNF in which BDNF molecules were incorporated into polyion complexes with block copolymers composed of safe and biocompatible diblock copolymers of (poly(ethylene glycol) (PEG) and poly(L-glutamate) (PGA) to increase CNS delivery. We have previously validated similar technology for the CNS delivery of an active antioxidant enzyme (Jiang et al., 2015). The PEG-PGA block copolymers used in this nanoparticle formulation has been used in preclinical and clinical trials to treat cancer, among other diseases ((Danson et al., 2004; Sahay et al., 2010; Valle et al., 2011). This delivery system is therefore very clinically relevant and could be used in stroke patients to improve functional recovery. In this work, the effectiveness of this novel nano-formulation of BDNF was examined. Both neuroprotective and neuro-restorative potential (an improvement in behavioral outcome without a change in infarct size) were examined after experimental stroke in mice.

METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center and were performed in accordance with National Institutes of Health guidelines. C57Bl/6 male mice (8–10 weeks; 20–25g) were purchased from Jackson Laboratories (Bar Harbor, ME). After arrival, mice were acclimatized in the animal care facility for at least 2 weeks and were maintained in an ambient temperature and humidity controlled vivarium with free access to food and water ad libitum. After gross behavioral examination all mice underwent 60 minutes of MCAo and were randomly assigned to one of three treatment conditions and one of three treatment time groups. Mice in Group A (early treatment) were treated twice with BDNF nano-particles (nano-BDNF) (250µg/kg i.v; n=6/group) or saline at 3 and 24 hours after MCAo using lateral tail vein. Mice in Groups B (intermediate treatment) and C (delayed treatment) were treated with saline, nano-BDNF, or native-BDNF (250µg/kg i.v., n=4–8/group) at 6 and 24 hours or 12 and 24 hours after MCAo, respectively. The amount of 250 µg/kg in our dose refers to the pure BDNF protein in the nanoformulation.

Additionally, a sub-cohort of mice in the delayed treatment group (Group D) underwent two additional behavioral tests, a memory test (Novel Object Recognition Test) on days 7 and 14 and a depression assessment (Tail Suspension Test) on day 14. All mice were sacrificed on day 15. Brains were used for cresyl violet staining, western blot analysis, or ELISA. Neurological deficit scores were recorded on days 0, 1, 3, 7, and 14. Serum samples were also collected at sacrifice and used for ELISA (See Figure 1). STAIR and RIGOR guidelines were followed in this study (Lapchak et al., 2013).

Stroke model

Focal transient cerebral ischemia was induced by a 60-minute right middle cerebral artery occlusion (MCAo) under Isoflurane anesthesia followed by reperfusion and 15 day survival as described previously (Verma et al., 2014). Briefly, a midline ventral neck incision was made and unilateral right MCAo was performed by advancing a 6.0 silicone rubber-coated

monofilament (Doccol Corporation, CA) 10–11 mm from the internal carotid artery bifurcation via an external carotid artery stump. Rectal temperatures were monitored with a temperature control system (Fine science tools, Canada) and were maintained at ~37 °C during surgery with an automatic heating pad. Laser Doppler Flowmetry (DRT 4/Moor Instruments Ltd, Devon, UK) was used to measure cerebral blood flow, and to confirm occlusion (reduction to 15% of baseline cerebral blood flow) and reperfusion. All animals were fed with wet mash for 1 week after surgery to ensure adequate nutrition for chronic endpoints, as animals have rearing deficits after stroke. Additionally, a daily subcutaneous injection of normal saline (volume=1% v/w) was given to all animals for 1 week.

Cresyl violet staining for Tissue Atrophy

Animals in groups A, B, and C were sacrificed 15 days after stroke surgery with an overdose of Avertin (250 mg/kg i.p). After blood collection by cardiac puncture, mice underwent trans-cardiac perfusion using cold PBS followed by 4% paraformaldehyde. Brains were then fixed overnight and placed in cyroprotectant (30% sucrose in PBS) for 72 hours before processing. Brains were then cut into 30-µm free-floating sections using a freezing microtome and every eighth slice was mounted and stained with cresyl violet. These 30-µm sections were then used for tissue atrophy calculation. Loss of brain tissue was determined by measuring the amount of tissue atrophy in the ipsilateral hemisphere. Tissue atrophy was calculated using the following formula: % tissue atrophy = (Total ipsilateral tissue/total contralateral tissue) × 100 (Verma et al., 2014). Data analysis was performed by an investigator blinded to the experimental cohort.

Western blot Analysis

Animals in group D were sacrificed 15 days after stroke surgery with an overdose of Avertin (250 mg/kg i.p) and blood was collected from the right ventricle. Mice underwent cervical dislocation followed by the rapid removal of the brain. The frontal cortical region of the right (ischemic) hemisphere was separated and homogenized as described previously (22). Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL) and subjected to Western Blotting as previously described (22). A total of 20 µg of protein was loaded into each well. Protein samples were resolved on 4% to 15% SDS electrophoresis gels and transferred to a PVDF (polyvinylidene difluoride membrane). We examined the differences in expression patterns of myelin basic protein (MBP) (1:1000 Abcam), TrkB (1:1000 Santa Cruz) and actin (1:500, Sigma) in mice treated with nano-BDNF, native-BDNF, and saline. Densitometry of Western blotting images (n= 5/ group) was performed with computer software (Image J).

ELISA

Blood samples (Group D – see collection method above) were spun at 10,000g for 10 minutes at 4°C; serum supernatant was collected. Both serum (no dilution factor; n=3–6/ group) and homogenized brain tissue (dilution factor: 1:2; n=3/group) were analyzed for BDNF levels using a BDNF (Mouse) ELISA Kit (Abnova).

Neurological deficit

The neurological deficit score (NDS) is a crude assessment of post-stroke behavioural recovery. ND scores, ranging from 0 to 4, were recorded on days 0, 1, 3, 7, and 14 post-MCAo (Liu et al., 2009). Our standard scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling (Venna et al., 2014).

Novel Object Recognition Task (NORT)

The NORT is used to evaluate cognition, particularly recognition memory, in rodent models of CNS disorders (Antunes and Biala, 2012). This test is based on the tendency of mice to spend more time exploring a novel object than a familiar one. This preference is used to assess intact recognition memory. Mice were placed in the behavioral room for an hour prior to testing to allow acclimatization. During habituation animals were allowed to explore an empty arena for at least 10 minutes. Twenty-four hours after habituation, animals were exposed to the familiar arena with 2 identical objects placed at an equal distance for 10 minutes (trial phase). If the total time of exploration of these objects was greater than 20 seconds, these mice qualified for the experimental test (Leger and Massoud, 2013) which was conducted 2 hours after the trial. One of the objects from the trial was replaced with a novel object. Mice were then allowed to explore the test arena for 10 minutes. The experiment was recorded using a digital video camera (JVC Everio, Victor Company, Japan) by a trained observer. A discrimination index (DI) was calculated by using the formula $DI = (T_N - T_F) / (T_N + T_F)$, where T_N = time spent exploring the novel object and T_F = time spent in exploring of familiar objects. The NORT was performed on days 7 and 14 after stroke and analyzed by an experimenter blinded to treatment. Different novel objects were used each week; the arena was cleaned between tests to remove olfactory cues.

Tail Suspension test (TST)

The tail suspension test (TST) was performed as described previously (Chatterjee et al., 2012) with minor modifications. Mice were placed in the behavioral room for an hour prior to testing to allow acclimatization. Briefly, the mice were individually suspended from the tail suspension apparatus, 60 cm above the surface of the table. The experiment was recorded for six minutes using a digital video camera (JVC Everio, Victor Company, Japan). A trained observer who was blinded to the treatment conditions then recorded the duration of immobility. The mouse was considered immobile in the absence of initiated movement. In general, an immobile mouse will appear to hang passively unless it has retained momentum from a movement immediately prior to the current bout of immobility. The testing apparatus was cleaned between trials to remove olfactory cues. Due to the potential stress induced by the TST, this was only performed once, on day 14, the day prior to sacrifice.

Statistics

All data were analyzed and expressed as means $\pm$ S. E. M. The non-parametric NDS in the 3-hour cohort was analyzed via Mann-Whitney U test. Tissue atrophy in the 3-hour cohort was analyzed by a T test. Tissue atrophy for the 6 and 12-hour cohort, as well as NORT,

TST, western blot, and ELISA data, were analyzed by a one-way ANOVA with a Newman-Keuls post hoc test to correct for multiple comparisons. A probability value of $p < 0.05$ was considered to be statistically significant.

Nano-BDNF

We prepared the nano-BDNF formulation by polyion complexation of human recombinant BDNF (Peprotech, Rocky Hill, NJ) with PEG(5 kDa)-PGA(9 kDa) diblock copolymer (Alamanda Polymers, Huntsville, AL) in aqueous solution in phosphate buffered saline, pH 7.4 (Fig. 2). Dynamic light scattering (DLS) revealed that the complex particle size is 95 nm with a relatively small polydispersity index (PDI) of 0.165 (measured at 0.1 mg/mL BDNF in de-ionized water). This reaction between charged proteins with doubly hydrophilic block copolymers with ionic and nonionic blocks has been well characterized (Harada and Kataoka, 1998, 1999) and applied as protein delivery tools (Jiang et al., 2016; Jiang et al., 2015; Manickam et al., 2012). The formulation was stored in the solution form at 4 °C before injection. The particle size did not change over the observation period of several weeks.

Radioactive Labeling of BDNF

BDNF and bovine serum albumin (BSA) were labeled with iodine by chloramine-T method (Yi et al., 2014). Briefly, BDNF was mixed with 1 mCi of Na^{125}I or Na^{131}I (Perkin Elmer) and 10 μg of chloramine-T in phosphate buffer (0.25 M, pH 7.5) for 60 s. Labeled protein was purified by Illustra Nap-5 columns (Life technologies) and collected in tubes pretreated with 1% BSA in PBS to prevent nonspecific adsorption. The iodine association (iodine in labeled sample/total iodine) was determined by trichloroacetic acid precipitation method (Yi et al., 2014). Briefly, 1 μl of purified samples was mixed with 0.5 ml of 1% BSA in PBS and 0.5 ml of 30% trichloroacetic acid, and then centrifuged at 5400 g for 10 min. The resulted pellet and supernatant were counted on r-counter (PerkinElmer). The iodine association was calculated as the percentage of pellet radioactivity to total radioactivity. The iodine association for BSA/BDNF was higher than 85% and 98%, respectively. The ^{125}I -labeled BDNF was used for preparation of polyion complexes with PEG-PGA (^{125}I -labeled nano-BDNF). In this case the tubes for preparation of the complexes were pre-coated with PEG-PGA avoid the tracer adsorption on the walls.

Pharmacokinetics studies

The studies of pharmacokinetics of the BDNF and nano-BDNF were carried out in CD-1 male mice (8 to 10 weeks of age) (Charles River Laboratories, Wilmington, Mass) as described previously (Yi et al., 2014). Briefly mice were be anesthetized with urethane and then given an injection into the tail vein of ^{125}I -BDNF or ^{125}I -nano-BDNF (3×10^5 cpm/mouse) in 0.2 ml of saline; the injection also contained ^{131}I -labeled BSA (3×10^5 cpm/mouse) that served a measure the brain's vascular space. The blood and whole brain were collected at different time points and measured in a gamma counter (3 mice per time point). As ^{131}I and ^{125}I are distinguishable in our gamma counter, we can measure cytokine and albumin uptake in the same animal. The volume of distribution (Vd), the serum half-life ($t_{1/2}$), the brain/serum ratio ($\mu\text{L/g}$), the % of the injected dose in a ml of serum (%Inj/ml) and taken up per g of brain (%Inj/g), the Area Under the Curve (AUC) in serum and brain, and

the unidirectional influx rate (K_i , [$\mu\text{l/g}\cdot\text{min}$]) were determined by multiple-time regression (Blasberg et al., 1983; Patlak et al., 1983).

RESULTS

BDNF nano-particles reduce tissue atrophy and improve neurological deficit scores

In our first experiment, we examined the neuroprotective potential of nano-BDNF. Mice were treated with nano-BDNF or saline at 3 and 24 hours after MCAo (Group A). We found a significant reduction in the amount of tissue lost ($31.02\pm 4.89\%$ vs. $41.33\pm 2.26\%$; $p=0.0062$) after nano-BDNF treatment (Fig. 2B). Moreover, nano-BDNF treated mice also showed earlier recovery of their neurological deficits on day 3 (Fig. 3C and 3D).

Next, the therapeutic window of nano-BDNF was assessed. We also performed a direct comparison between nano-BDNF and native-BDNF formulations. The first treatment dose (nano-BDNF, native-BDNF, or saline) was delayed until 6 hours after ischemic onset (Group B). Nano-BDNF significantly reduced tissue loss after MCAo compared to native-BDNF treatment ($11.89\pm 12.82\%$ vs. $36.40\pm 5.97\%$) [$F(2, 12) = 8.256$, $p = 0.00432$]; no difference in tissue loss was seen between saline and native-BDNF treatment (Fig. 4A). Similar to 3 hour treatment, 6 hour treatment with nano-BDNF showed a positive trend in NDS. However, results were not significant ($P=0.13$, 0.17 and 0.2302 at Day 3, 7 and 14 respectively between saline and nano BDNF cohort B). In a third cohort (Group C and D), the first dose of nano-BDNF was further delayed until 12 hours after ischemic onset. In third cohort, however, no significant differences in tissue atrophy (Fig 4B) or NDS was found (Day 7: 1.43 ± 0.23 vs 1.16 ± 0.3 vs 0.85 ± 0.41 and day 14: 1.21 ± 0.25 vs 1 ± 0.32 vs 0.8 ± 0.43 respectively in saline vs native vs nano-BDNF).

Delayed treatment with Nano-BDNF formulation improves memory and depressive behavior

BDNF treatment has been found to improve memory after stroke and have general antidepressant effects (Schmidt et al., 2011; Sirianni et al., 2010; Zhang et al., 2012). Given that we found no neuroprotective effects of nano-BDNF 12 hours after ischemia, we decided to evaluate an additional cohort of animals (Group D) for chronic behavioral changes after delayed BDNF treatment (treated at 12 and 24 hours post MCAo). This paradigm allows us to separate neuroprotective from neuro-restoration as infarct damage was equivalent in the treatment groups. Nano-BDNF treatment led to improved learning and memory at day 7 after MCAo compared to saline treated mice ($p<0.05$), as shown by a significantly higher discrimination Index (DI) [$F(2, 12) = 4.224$, $p = 0.0468$], while native-BDNF treatment led to intermediate effects (Fig. 5). It should be noted that nano-BDNF treated mice also had a higher DI at day 14, but this was not statistically significant, perhaps due to the repeated testing. In addition to an improvement in memory, delayed nano-BDNF treatment led to a reduction in depressive-like behavior as shown by significantly reduced immobility in the tail suspension test when compared to saline ($p<0.05$) and native-BDNF treated mice ($p<0.01$) [$F(2, 12) = 9.953$, $p = 0.0034$] (Fig. 6). These behavioral improvements were seen despite the lack of gross histological change.

Nano BDNF treatment increases MBP expression and brain BDNF levels

The effect of nano-BDNF treatment on MBP and Trk-B expression in the frontal cortical region of right (ipsilateral) hemisphere was assessed in mice from the delayed treatment cohort (Group D). We found no significant difference in TrkB expression between groups (data not shown). We did, however, find that nano-BDNF treatment led to significantly increased expression of MBP compared to saline treatment (4.36 ± 0.20 vs. 2.56 ± 0.08 ; $p < .01$); native-BDNF treatment led to a less significant increase in MBP levels when compared to saline treated animals (3.58 ± 0.11 vs. 2.56 ± 0.08); [$F(2, 12) = 41.52$, $p = 0.0065$] (Fig. 7B). Mice treated with nano-BDNF had significantly higher brain BDNF levels than saline treated mice (1339.79 ± 40.39 vs. 1145.08 ± 35.52) [$F(2, 9) = 8.165$, $p = 0.0148$] (Fig 8A), however, BDNF levels were not significantly different between native-BDNF and saline treated mice. We found no differences in serum BDNF levels between groups (Fig. 8B).

To assess whether our nanoformulation increases the entry of the BDNF protein to the brain we characterized the pharmacokinetics of the free BDNF and nano-BDNF after IV administration. In this study, BDNF was labeled by I^{125} and then formulated with PEG-PGA. These BDNF forms were administered IV into CD-1 mice and the radioactivity levels in the blood and brain, were measured at different time points (Fig. 9). The nano-BDNF shows similar serum clearance as BDNF ($t_{1/2}$ 15.6 min vs. 15.1 min). Importantly, the brain pharmacokinetics profile of nano-BDNF was drastically improved as demonstrated by: 1) increased influx rate across the blood brain barrier, measured by K_i at $0.84 \mu\text{L/g.min}$ (the slope); whereas BDNF was rapidly effluxed from brain to blood; 2) increased uptake compared to that of BDNF by over 6 times, calculated by AUC ($2.96 (\% \text{inj/g}) \times (\text{min})$ for nano-BDNF vs 0.54 for BDNF) over 10 min following IV injection; and 3) crossing the BBB and accumulation in brain parenchyma.

DISCUSSION

This study examined the neuroprotective and neuro-restorative efficacy of a novel nano-BDNF formulation. Despite the fact that BDNF administration after stroke can reduce infarct size, limit post-stroke depressive behavior, enhances post-stroke sensorimotor recovery, cognition and neurogenesis in animal models, its limited brain bioavailability and short serum half-life have limited its usefulness in a clinical setting. In this study, we examined the efficacy of “nano-BDNF” in which BDNF molecules are incorporated into polyion complexes with block copolymers composed of safe and biocompatible polymers, poly (ethylene glycol) (PEG) and poly(L-glutamate) (PGA). We have previously validated the polyion complex technology in the delivery of an active antioxidant enzyme to the brain in an animal model of cerebral ischemia (Jiang et al., 2015). In this study, we evaluated nano-BDNF as a potential therapeutic agent for the treatment of brain injury in an animal model of stroke.

Although previous studies have shown the efficacy of BDNF in improving post-stroke recovery in animal models (Clarkson et al., 2015; Yu et al., 2013), BDNF was given quite quickly after stroke onset, a therapeutic time-window that is often not practical in clinical situations. In this study, extended therapeutic windows were evaluated, with treatment

delayed for over 12 hours after stroke onset. We first showed the efficacy of early nano-BDNF treatment in reducing tissue loss. We next examined the efficacy of nano-BDNF at a later first dose time point, 6 hours. At this time point we also added a native-BDNF-treatment as an additional control to rule out any contribution of the peripheral effects of BDNF treatment and to allow us to directly compare native BDNF with the novel nano-BDNF formulation. We found that nano-BDNF treatment led to significantly reduced tissue damage when compared to native-BDNF, confirming the improved efficacy of the novel nanoparticle formulation.

We also examined the efficacy of nano-BDNF treatment at an even more delayed first dose time point, 12 hours after stroke onset. Delayed nano-BDNF treatment even as late as 12 hours following stroke increased MBP expression and BDNF levels in the brain 15 days after drug administration. As the increased brain accumulation of BDNF after IV administration of nano-BDNF appears to be short-lived (minutes) we assume that any differences in brain BDNF levels at 15 days were due to differences in endogenous activation of BDNF, and not due to residual BDNF from the initial tail vein injection.

Moreover, 12 hour nano-BDNF also reduced post-stroke depressive phenotypes, and improved cognitive deficits. Many previous studies have found post-stroke behavioral improvement even in the absence of histological changes especially at chronic endpoints (Johansson, 1996; Johansson and Ohlsson, 1996; Yamamoto et al., 1989), which was seen in this study. Gain or loss of BDNF has been shown to lead to an increase or decrease in MBP expression respectively (Djalali et al., 2005). The upregulation of MBP and brain BDNF levels after nano-BDNF treatment supports the hypothesis that the behavioral recovery that we observed was due to nano-BDNF and this formulation is superior to that of native-BDNF. Taken together, these results suggest that delayed treatment of nano-BDNF beyond 6 hours is not neuroprotective, as infarct size was equivalent, however nano-BDNF continues to have important neuro-restorative effects that are seen weeks after drug administration. Targeting the recovery phase of stroke may lead to enhancement of functional outcomes that are independent of infarct size.

The benefits of BDNF treatment have been linked to enhancements in neurogenesis, brain repair, neuronal activity, cell survival as well as remyelination after white matter injury in subcortical stroke models (Hermann and Chopp, 2012; Murphy and Corbett, 2009). However, the exact mechanism by which BDNF is restorative is not well understood. Our observation that nano-BDNF treatment increases the expression of MBP even after delayed treatment is consistent with previous findings (Ramos-Cejudo et al., 2015). As enhancing MBP can have positive effects on both cognitive and motor skills (Lu et al., 2013; McKenzie et al., 2014), the increase in MBP expression may be partially responsible for the behavioral improvements seen in the cohorts of mice treated with nano-BDNF.

We do not report here on the mechanism of action of nano-BDNF as these studies were focused on determining the translational value of these novel nanoparticles as a therapy for stroke. We and others have previously shown that enhanced behavioral recovery in stroke mice is secondary to an enhancement in neurogenesis (Schabitz et al., 2007; Venna et al., 2014). Specifically, one study has found that behavioral improvements after stroke paralleled

changes in BDNF levels and neurogenesis in the SVZ (Schabitz et al., 2007; Venna et al., 2014). Additionally, increased neurogenesis has been found to lead to improvements in object recognition, spatial learning, and overall cognitive performance, and also reduces depressive-like behaviors (Hill et al., 2015; Sahay et al., 2011). In addition to enhancing neurogenesis and increasing MBP expression, BDNF also increases dendritic spine density and cAMP response element-binding (CREB) expression in astrocytes (Pilakka-Kanthikeel et al., 2013). Increases in both dendritic spine density and CREB expression enhance memory consolidation (Barco et al., 2003; Kasai et al., 2010). Moreover, the upregulation of CREB levels is associated with improvement of depressive behavior in rats (Itoh et al., 2004). Therefore, BDNF's effect on CREB expression, dendritic spine density, MBP expression, and/or neurogenesis, may all be potential mechanism through which our nano-BDNF formulation led to improvements in learning/cognition and depressive phenotype in delayed treatment cohort. These will be examined in future studies now the efficacy of this treatment has been confirmed. Additional studies are needed to assess the effectiveness of delayed nano-BDNF in females and aged animals to move this agent into therapeutic trials.

SUMMARY/LIMITATIONS

The purpose of this study was to examine the efficacy of a novel nanoparticle formulation of BDNF in improving post-stroke recovery. Administration of nano-BDNF improved neuropathological and neurobehavioral outcomes after stroke. Early and intermediate treatment (3 and 6 hours) after stroke onset led to reduced tissue atrophy. Delayed (12 hour) treatment led to increased expression of MBP and improvements in memory and depressive phenotypes beyond that seen with native BDNF or saline treatment. Therefore, this nano-formulation of BDNF has novel and additional benefits beyond that of BDNF, and has a wide therapeutic window. Despite the important findings with our BDNF nano formulation, there were several limitations in this initial study. First, we used a dose that was predicted based on in vitro studies, nano-BDNF pharmacokinetics studies in healthy animals and prior studies using exogenous non-nano BDNF formulations (250 µg/kg), which was found effective. Optimization of our dosing regimen and route are needed and pharmacokinetics studies of BDNF forms that would need to be carried out using the MCAo model. Second, we used relatively low numbers of animals in these studies, which were sufficient to show significant histological differences between groups (the primary outcome variable of the present study). In all of the infarct studies we perform in our laboratory, we undertake an interval analysis after 5–6 animals to refine the animal numbers needed to reach significance (or for futility) using power analysis (G*power). In this case, significance was seen with relatively small numbers, speaking to the potential of nano-BDNF to reduce injury. Our main aim for the study was to validate this nano-formulation for in vivo use. Our initial findings are very encouraging that this formulation of BDNF will have considerable potential to reduce stroke-induced tissue damage and its related disability. Moreover, we hypothesize that the small sample size may have accounted for the lack of significance in our intermediate and delayed treatment groups. We did not, however, increase the sample size given that we already found significant differences in tissue atrophy in this group. As NDS is known to be relatively insensitive after the first few days after stroke in young animals (Li et al., 2004), we therefore used more sensitive behavioral tests at longer time

points (e.g. TST and NORT), for which we found significant differences. A separately standing question relates to the mechanism of release of the active BDNF from the nano-BDNF polyion complex which is a of an ongoing study that will be published elsewhere. Here we would like to point out that nano-BDNF is similar to the DNA/polycation complexes (“polyplexes”) that have been extensively studied. Such complexes are very stable but have a dynamic character and can undergo very rapid (seconds) cooperative reactions of polyion interchange resulting in displacement of one polyion by another which believed to be responsible for the release and subsequent processing of the DNA from polyplexes (Chelushkin et al., 2008; Kabanov and Kabanov, 1995). Clearly such processes are also responsible for the formation and subsequent transitions of nano-BDNF especially upon its interaction with the high affinity TrkB receptor. Future work will further develop this promising agent using animals with common co-morbidities (aged, hypertensive, etc.) and utilizing large animal models of stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Alexander V Kabanov is a founder, shareholder and board member of Neuronano Pharma Inc. that develops nano-BDNF and other nanoformulations for protein delivery to the brain.

References

- Allen AP, Naughton M, Dowling J, Walsh A, Ismail F, Shorten G, Scott L, McLoughlin DM, Cryan JF, Dinan TG, Clarke G. Serum BDNF as a peripheral biomarker of treatment-resistant depression and the rapid antidepressant response: A comparison of ketamine and ECT. *Journal of affective disorders*. 2015; 186:306–311. [PubMed: 26275358]
- Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing*. 2012; 13(2):93–110. [PubMed: 22160349]
- Barco A, Pittenger C, Kandel ER. CREB, memory enhancement and the treatment of memory disorders: promises, pitfalls and prospects. *Expert opinion on therapeutic targets*. 2003; 7(1):101–114. [PubMed: 12556206]
- Bejot Y, Mossiat C, Giroud M, Prigent-Tessier A, Marie C. Circulating and brain BDNF levels in stroke rats. Relevance to clinical studies. *PloS one*. 2011; 6(12):e29405. [PubMed: 22195050]
- Blasberg RG, Fenstermacher JD, Patlak CS. Transport of alpha-aminoisobutyric acid across brain capillary and cellular membranes. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 1983; 3(1):8–32.
- Chatterjee M, Jaiswal M, Palit G. Comparative evaluation of forced swim test and tail suspension test as models of negative symptom of schizophrenia in rodents. *ISRN psychiatry*. 2012; 2012:595141. [PubMed: 23738205]
- Chelushkin PS, Lysenko EA, Bronich TK, Eisenberg A, Kabanov VA, Kabanov AV. Polyion complex nanomaterials from block polyelectrolyte micelles and linear polyelectrolytes of opposite charge. 2. Dynamic properties. *The journal of physical chemistry. B*. 2008; 112(26):7732–7738. [PubMed: 18533694]

- Clarkson AN, Parker K, Nilsson M, Walker FR, Gowing EK. Combined ampakine and BDNF treatments enhance poststroke functional recovery in aged mice via AKT-CREB signaling. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*. 2015; 35(8):1272–1279.
- Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, Brampton M, Halbert G, Ranson M. Phase I dose escalation and pharmacokinetic study of pluronic polymerbound doxorubicin (SP1049C) in patients with advanced cancer. *British journal of cancer*. 2004; 90(11):2085–2091. [PubMed: 15150584]
- Djalali S, Holtje M, Grosse G, Rothe T, Stroth T, Grosse J, Deng DR, Hellweg R, Grantyn R, Hortnagl H, Ahnert-Hilger G. Effects of brain-derived neurotrophic factor (BDNF) on glial cells and serotonergic neurones during development. *Journal of neurochemistry*. 2005; 92(3):616–627. [PubMed: 15659231]
- Fluri F, Schuhmann MK, Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. *Drug design, development and therapy*. 2015; 9:3445–3454.
- Harada A, Kataoka K. Novel polyion complex micelles entrapping enzyme molecules in the core: Preparation of narrowly-distributed micelles from lysozyme and poly(ethylene glycol)-poly(aspartic acid) block copolymer in aqueous medium. *Macromolecules*. 1998; 31(2):288–294.
- Harada A, Kataoka K. Novel polyion complex micelles entrapping enzyme molecules in the core. 2. Characterization of the micelles prepared at nonstoichiometric mixing ratios. *Langmuir*. 1999; 15(12):4208–4212.
- Hermann DM, Chopp M. Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *The Lancet. Neurology*. 2012; 11(4):369–380. [PubMed: 22441198]
- Hill AS, Sahay A, Hen R. Increasing Adult Hippocampal Neurogenesis is Sufficient to Reduce Anxiety and Depression-Like Behaviors. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2015; 40(10):2368–2378. [PubMed: 25833129]
- Hong SH, Khoutorova L, Bazan NG, Belayev L. Docosahexaenoic acid improves behavior and attenuates blood-brain barrier injury induced by focal cerebral ischemia in rats. *Experimental & translational stroke medicine*. 2015; 7(1):3. [PubMed: 25642315]
- Itoh T, Tokumura M, Abe K. Effects of rolipram, a phosphodiesterase 4 inhibitor, in combination with imipramine on depressive behavior, CRE-binding activity and BDNF level in learned helplessness rats. *European journal of pharmacology*. 2004; 498(1–3):135–142. [PubMed: 15363987]
- Jiang Y, Arounleut P, Rheiner S, Bae Y, Kabanov AV, Milligan C, Manickam DS. SOD1 nanozyme with reduced Toxicity and MPS accumulation. *Journal of controlled release : official journal of the Controlled Release Society*. 2016
- Jiang Y, Brynskikh AM, D SM, Kabanov AV. SOD1 nanozyme salvages ischemic brain by locally protecting cerebral vasculature. *Journal of controlled release : official journal of the Controlled Release Society*. 2015; 213:36–44. [PubMed: 26093094]
- Johansson BB. Environmental influence on outcome after experiment brain infarction. *Acta neurochirurgica*. 1996; (Supplement 66):63–67.
- Johansson BB, Ohlsson AL. Environment, social interaction, and physical activity as determinants of functional outcome after cerebral infarction in the rat. *Experimental neurology*. 1996; 139(2):322–327. [PubMed: 8654535]
- Kabanov AV, Kabanov VA. DNA complexes with polycations for the delivery of genetic material into cells. *Bioconjugate chemistry*. 1995; 6(1):7–20. [PubMed: 7711106]
- Kasai H, Fukuda M, Watanabe S, Hayashi-Takagi A, Noguchi J. Structural dynamics of dendritic spines in memory and cognition. *Trends in neurosciences*. 2010; 33(3):121–129. [PubMed: 20138375]
- Lapchak PA, Zhang JH, Noble-Haeusslein LJ. RIGOR guidelines: escalating STAIR and STEPS for effective translational research. *Translational stroke research*. 2013; 4(3):279–285. [PubMed: 23658596]
- Lee HJ, Lim IJ, Lee MC, Kim SU. Human neural stem cells genetically modified to overexpress brain-derived neurotrophic factor promote functional recovery and neuroprotection in a mouse stroke model. *Journal of neuroscience research*. 2010; 88(15):3282–3294. [PubMed: 20818776]

- Leger GC, Massoud F. Novel disease-modifying therapeutics for the treatment of Alzheimer's disease. Expert review of clinical pharmacology. 2013; 6(4):423–442. [PubMed: 23927670]
- Lenzi GL, Altieri M, Maestrini I. Post-stroke depression. Revue neurologique. 2008; 164(10):837–840. [PubMed: 18771785]
- Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD, McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. Experimental Neurology. 2004; 187(1):94–104. [PubMed: 15081592]
- Liu S, Zhen G, Meloni BP, Campbell K, Winn HR. Rodent Stroke Model Guidelines for Preclinical Stroke Trials (1st Edition). Journal of experimental stroke & translational medicine. 2009; 2(2):2–27. [PubMed: 20369026]
- Lokk J, Delbari A. Management of depression in elderly stroke patients. Neuropsychiatric disease and treatment. 2010; 6:539–549. [PubMed: 20856917]
- Lu PH, Lee GJ, Tishler TA, Meghpara M, Thompson PM, Bartzokis G. Myelin breakdown mediates age-related slowing in cognitive processing speed in healthy elderly men. Brain and cognition. 2013; 81(1):131–138. [PubMed: 23195704]
- Mang CS, Campbell KL, Ross CJ, Boyd LA. Promoting neuroplasticity for motor rehabilitation after stroke: considering the effects of aerobic exercise and genetic variation on brain-derived neurotrophic factor. Physical therapy. 2013; 93(12):1707–1716. [PubMed: 23907078]
- Manickam DS, Brynskikh AM, Kopanic JL, Sorgen PL, Klyachko NL, Batrakova EV, Bronich TK, Kabanov AV. Well-defined cross-linked antioxidant nanozymes for treatment of ischemic brain injury. Journal of controlled release : official journal of the Controlled Release Society. 2012; 162(3):636–645. [PubMed: 22902590]
- McKenzie IA, Ohayon D, Li H, de Faria JP, Emery B, Tohyama K, Richardson WD. Motor skill learning requires active central myelination. Science. 2014; 346(6207):318–322. [PubMed: 25324381]
- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB. American Heart Association Statistics, C., Stroke Statistics, S. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation. 2016; 133(4):e38–e360. [PubMed: 26673558]
- Murphy TH, Corbett D. Plasticity during stroke recovery: from synapse to behaviour. Nature reviews. Neuroscience. 2009; 10(12):861–872. [PubMed: 19888284]
- O'Keefe LM, Doran SJ, Mwilambwe-Tshilobo L, Conti LH, Venna VR, McCullough LD. Social isolation after stroke leads to depressive-like behavior and decreased BDNF levels in mice. Behavioural brain research. 2014; 260:162–170. [PubMed: 24211537]
- Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. Neuropharmacology. 1998; 37(12):1553–1561. [PubMed: 9886678]
- Patlak CS, Blasberg RG, Fenstermacher JD. Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 1983; 3(1):1–7.
- Pilakka-Kanthikeel S, Atluri VS, Sagar V, Saxena SK, Nair M. Targeted brain derived neurotrophic factors (BDNF) delivery across the blood-brain barrier for neuro-protection using magnetic nano carriers: an in-vitro study. PloS one. 2013; 8(4):e62241. [PubMed: 23653680]
- Ramos-Cejudo J, Gutierrez-Fernandez M, Otero-Ortega L, Rodriguez-Frutos B, Fuentes B, Vallejo-Cremades MT, Hernanz TN, Cerdan S, Diez-Tejedor E. Brain-derived neurotrophic factor administration mediated oligodendrocyte differentiation and myelin formation in subcortical ischemic stroke. Stroke; a journal of cerebral circulation. 2015; 46(1):221–228.
- Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature. 2011; 472(7344):466–470. [PubMed: 21460835]

- Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. *Journal of controlled release : official journal of the Controlled Release Society*. 2010; 145(3):182–195. [PubMed: 20226220]
- Schabitz WR, Steigleder T, Cooper-Kuhn CM, Schwab S, Sommer C, Schneider A, Kuhn HG. Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. *Stroke; a journal of cerebral circulation*. 2007; 38(7):2165–2172. [PubMed: 17510456]
- Schmidt HD, Shelton RC, Duman RS. Functional biomarkers of depression: diagnosis, treatment, and pathophysiology. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2011; 36(12):2375–2394. [PubMed: 21814182]
- Sirianni RW, Olausson P, Chiu AS, Taylor JR, Saltzman WM. The behavioral and biochemical effects of BDNF containing polymers implanted in the hippocampus of rats. *Brain research*. 2010; 1321:40–50. [PubMed: 20096671]
- Sullivan PG, Sebastian AH, Hall ED. Therapeutic window analysis of the neuroprotective effects of cyclosporine A after traumatic brain injury. *Journal of neurotrauma*. 2011; 28(2):311–318. [PubMed: 21142667]
- Synhaeve NE, Schaapsmeeders P, Arntz RM, Maaijwee NA, Rutten-Jacobs LC, Schoonderwaldt HC, Dorresteyn LD, de Kort PL, van Dijk EJ, Kessels RP, de Leeuw FE. Cognitive performance and poor long-term functional outcome after young stroke. *Neurology*. 2015; 85(9):776–782. [PubMed: 26245928]
- Valle JW, Armstrong A, Newman C, Alakhov V, Pietrzynski G, Brewer J, Campbell S, Corrie P, Rowinsky EK, Ranson M. A phase 2 study of SP1049C, doxorubicin in P-glycoprotein- targeting pluronics, in patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction. *Investigational new drugs*. 2011; 29(5):1029–1037. [PubMed: 20179989]
- Venna VR, Xu Y, Doran SJ, Patrizz A, McCullough LD. Social interaction plays a critical role in neurogenesis and recovery after stroke. *Translational psychiatry*. 2014; 4:e351. [PubMed: 24473442]
- Verma R, Friedler BD, Harris NM, McCullough LD. Pair housing reverses post-stroke depressive behavior in mice. *Behavioural brain research*. 2014; 269:155–163. [PubMed: 24793492]
- Yamamoto M, Tamura A, Kirino T, Hirakawa M, Shimizu M, Sano K. Effects of a new thyrotropin-releasing hormone analogue administered in rats 1 week after middle cerebral artery occlusion. *Stroke; a journal of cerebral circulation*. 1989; 20(8):1089–1091.
- Yasuhara T, Hara K, Maki M, Masuda T, Sanberg CD, Sanberg PR, Bickford PC, Borlongan CV. Dietary supplementation exerts neuroprotective effects in ischemic stroke model. *Rejuvenation research*. 2008; 11(1):201–214. [PubMed: 18260778]
- Yi X, Yuan D, Farr SA, Banks WA, Poon CD, Kabanov AV. Pluronic modified leptin with increased systemic circulation, brain uptake and efficacy for treatment of obesity. *Journal of controlled release : official journal of the Controlled Release Society*. 2014; 191:34–46. [PubMed: 24881856]
- Yu SJ, Tseng KY, Shen H, Harvey BK, Airavaara M, Wang Y. Local administration of AAV-BDNF to subventricular zone induces functional recovery in stroke rats. *PloS one*. 2013; 8(12):e81750. [PubMed: 24312581]
- Zavoreo I, Basic-Kes V, Bosnar-Puretic M, Demarin V. Post-stroke depression. *Acta clinica Croatica*. 2009; 48(3):329–333. [PubMed: 20055258]
- Zhang L, Chen X, Feng W, Cui Y, Xu S, Che Y. Enhancing effects of chronic lithium treatment on detour learning in chicks. *Biological trace element research*. 2012; 148(1):38–43. [PubMed: 22290294]

Highlights

1. Nano formulation of BDNF is both neuro-protective and neuro-restorative.
2. Early treatment with nano-BDNF reduce infarct after stroke
3. Delayed treatment ameliorates depressive behavior and memory loss.

Figure 1.

Experimental design. Middle cerebral artery occlusion (MCAO) was performed on day 0 and either nano-BDNF, native-BDNF, or saline was injected intravenously at the respective time points. Brain tissue samples were collected from all mice on day 15 and were analyzed for tissue atrophy (in Groups A, B, and C) and for western blot analysis (in Group D). Tissue loss was calculated by subtracting the ischemic hemisphere (damaged) volume, including ipsilateral ventricle, from non-ischemic volume, including contralateral ventricle. **A)** Early treatment group: Neurological deficit scores were collected on days 0, 1, 3, 7 and 14. **B)** Intermediate treatment group. **C)** Delayed treatment group. **D)** Additional delayed treatment sub-cohort: Due to the lack of any difference in tissue atrophy outcome in our 12 hour group (Group C), we performed additional behavioral tests, and western blot and ELISA analysis in an additional 12 hour cohort (Group D).

Figure 2.

Schematic illustration showing the spontaneous formation of BDNF nano particle in water upon simple mixing of the native BDNF and PEG-PGA block copolymer. BDNF electrostatically couples with the oppositely charged PGA chain and entraps into a particle core surrounded by a shell of uncharged water-soluble PEG.

Figure 3.

A) Representative images of cresyl violet stained coronal brain sections. **B)** Group A: Quantification of tissue atrophy. Mice treated with nano-BDNF formulation (3 and 24 hours post MCAO) had significantly reduced tissue loss compared to saline treated mice on day 7 of survival ($p=0.0062$; $n=5/\text{group}$). **(C)** Line graph showing the recovery trajectory of nano-BDNF and saline treated mice; on day 3 mice treated with nano-BDNF formulation (3 and 24 hours post MCAO) had significantly improved neurological deficit scores compared to saline on day 3 after MCAO. **(D)** Day 3 NDS. Bar graph.

Figure 4.

Groups B and C: Quantification of tissue atrophy. **A)** Mice treated with nano-BDNF 6 and 24 hours post MCAO had significantly reduced tissue loss compared to saline treated mice after 15 days of survival ($F(2, 12) = 8.256$, $p = 0.00432$; $n=5/\text{group}$). **B)** Delayed treatment with nano-BDNF (12 and 24 hours post MCAO) did not significantly reduce tissue loss compared to native-BDNF or saline treatment.

Figure 5.

Group C: Novel Object Recognition Test. Despite the lack of significant histological changes in infarct volume, nano-BDNF treated mice (12 and 24 hours post MCAO) had improved learning and memory on day 7, as shown by a significantly higher discrimination Index (DI), when compared to saline and native-BDNF treated mice ($[F(2, 12) = 4.224, p = 0.0468]$ ($n=5$ group)). BDNF treated mice also had a higher DI at day 14, but this was not significant.

Figure 6.

Group C: Tail Suspension Test. Despite the absence of changes in infarct volume, nano-BDNF treated mice (12 and 24 hours post MCAO) had significantly reduced immobility compared to saline ($p < .05$) and native ($p < .01$) treated mice [$F(2, 12) = 9.953$, $p = 0.0034$] ($n = 5/\text{group}$), signifying a reduced depressive phenotype.

Figure 7.

Group C: Western Blot analysis. **A)** Representative image. **B)** Nano treatment (12 and 24 hours post MCAO) led to a more significant increase in MBP levels ($p < .01$) compared to saline than did native-BDNF treated mice [$F(2, 12) = 41.52$, $p = 0.0065$] ($n = 5/\text{group}$). Two MBP specific bands were present between 17–22kDa.

Figure 8.

Brain and serum BDNF ELISA. **A)** Despite the lack of significant histological changes in infarct volume, nano-BDNF treated mice (12 and 24 hours post MCAO) had significantly higher brain BDNF levels than saline treated mice 15 days following MCAO ($[F(2, 9) = 8.165, p = 0.0148]$ ($n=4/\text{group}$); however, there were no significant differences between nano and native-BDNF treated mice or between saline and native-BDNF treated mice. **B)** There were no significant differences between groups in serum BDNF levels ($n=3-6/\text{group}$).

Figure 9.

Brain pharmacokinetics study shows that (A) nano-BDNF clears from the circulation similarly to native BDNF; (B) nano-BDNF displays net influx ($K_i = 0.84 \text{ uL/g.min}$) into the brain, whereas native BDNF displays a net efflux from brain to the blood; (C) consequently nano-BDNF displays higher brain uptake than native BDNF, as shown by AUC of 2.96 for nano-BDNF vs 0.54 for native BDNF. The native BDNF or nano-BDNF were injected IV at a single dose of $25 \text{ }\mu\text{g/kg}$. In Plot B, the slope and Y-intercept of the brain/serum ratio determine the K_i (influx rate) and V_i (initial volume of distribution) of the drug, respectively. The negative slope for the native BDNF (negative influx rate) means that there is efflux of native BDNF from the brain. In the case of nano-BDNF, the slope is positive suggesting that there is net influx. Since this is a single bolus injection experiment the ratio changes over time.