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Neuroprotective and neurorestorative effects of thymosin beta4 treatment initiated 6 hours post injury following traumatic brain injury in rats

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Abstract

Object—Thymosin beta 4 (Tβ4) is a regenerative multifunctional peptide. This study will test the hypothesis that Tβ4 treatment initiated 6 hours post-injury reduces brain damage and improves functional recovery in rats after traumatic brain injury (TBI).

Methods—TBI was induced by controlled cortical impact over the left parietal cortex. Young adult male Wistar rats with TBI were randomly divided into the following groups: 1) Saline group ($n=7$); 2) Tβ4-6 mg/kg group ($n=8$), and 3) Tβ4-30 mg/kg group ($n=8$). Tβ4 or saline was administered intraperitoneally starting at 6 hours post-injury and then repeated daily at 24 and 48 hours. An additional group of sham animals underwent surgery without injury ($n=6$). Sensorimotor function and spatial learning were assessed using a modified neurological severity score and Morris water maze tests, respectively. Animals were sacrificed 35 days after injury and brain sections processed to assess lesion volume, hippocampal cell loss, cell proliferation and neurogenesis after Tβ4 treatment.

Results—Compared to saline, Tβ4 treatment initiated 6 hours post-injury significantly improved sensorimotor functional recovery and spatial learning, reduced cortical lesion volume and hippocampal cell loss, and enhanced cell proliferation and neurogenesis in the injured hippocampus. The high dose of Tβ4 showed better beneficial effects compared to the low-dose treatment.

Conclusions—Tβ4 treatment initiated 6 hours post-injury provides both neuroprotection and neurorestoration after TBI, indicating that Tβ4 has promising therapeutic potential in TBI patients.

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Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: Xiong, Mahmood, ZG Zhang, Morris, Chopp. Acquisition of data: Xiong, Meng, Y Zhang. Analysis and interpretation of data: Xiong, Mahmood, ZG Zhang, Chopp. Drafting the article: Xiong, Chopp. Critically revising the article: Xiong, Chopp. Reviewed final version of the manuscript and approved it for submission: all authors.

These data warrant further investigation of the optimal dose and therapeutic window of T β 4 treatment for TBI and the associated underlying mechanisms.

Keywords

neuroprotection; neurogenesis; rat; thymosin beta 4; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in young people.¹ Despite advances in preclinical research as well as improved clinical intensive care in recent years, no effective pharmacological therapy is available to promote functional recovery after TBI.^{38,45,57} TBI clinical trials targeting a single pathophysiological pathway have failed; a successful therapy may require targeting multiple injury pathways.^{38,45} There is a compelling need to develop effective therapies to improve functional outcome after TBI.

Thymosin beta 4 (T β 4), a peptide of 43-amino acids, was first isolated from calf thymus tissue and subsequently found in all mammalian species studied.⁴¹ The major intracellular function of T β 4 is to sequester G-actin monomers, which is necessary for cell motility and organogenesis.¹² T β 4 is also a regenerative multifunctional peptide, which inhibits inflammation and apoptosis,⁵⁴ and promotes tissue repair in skin,⁴⁸ cornea,¹⁸ and heart.^{5,56} T β 4 is an essential paracrine factor of endothelial progenitor cells,²³ which mediates cardioprotection.⁶⁶ T β 4 promotes angiogenesis after ischemic injury.^{61,63} The safety profile of T β 4 has been established in animals and humans.⁵⁸

T β 4 exists in the nervous system and is involved in many cellular processes including mobility, axonal path-finding, neurite formation, proliferation and neuronal survival.^{50,80} Our previous study demonstrates that T β 4 improves neurological functional recovery in rodents with experimental autoimmune encephalomyelitis and stroke.^{42,82} Our recent study shows that T β 4 treatment initiated 24 hours post-TBI does not reduce cortical lesion volume but reduces hippocampal cell loss and promotes endogenous neurorestorative effects, which potentially contribute to improved functional recovery.⁷⁷ However, the therapeutic efficacy of early T β 4 treatment for TBI has not been investigated. Although human TBI is a complex and multifaceted disease, pharmacological compounds delivered in a clinically-relevant time window may improve the outcome of TBI patients.³⁷ In the present study, we test the hypothesis that T β 4 initiated 6 hours post-injury provides neuroprotection by reducing cortical lesion volume and hippocampal cell loss, promotes brain remodeling by enhancing neurogenesis, and improves functional recovery in rats subjected to moderate TBI induced by unilateral controlled cortical impact.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Health System. All functional tests were performed by investigators blinded to the treatment status.

TBI Model

A controlled cortical impact (CCI) model of TBI in the rat was utilized for the present study.^{14,36} Young adult male Wistar rats (336.6 ± 15.2 g) were anesthetized with 35 mg/100 g body weight chloral hydrate intraperitoneally. Rectal temperature was maintained at 37°C using a feedback-regulated water-heating pad. Rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The contralateral craniotomy allowed for movement of

cortical tissue laterally. The dura mater was kept intact over the cortex. Injury was delivered by impacting the left (ipsilateral) cortex with a pneumatic piston containing a 6-mm-diameter tip at the rate of 4 m/second and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer.

Experimental Groups and Treatment

TBI was induced by CCI over the left parietal cortex. Young adult male Wistar rats with TBI were randomly divided into 3 groups: 1) Saline group ($n = 7$); 2) T β 4-6 mg/kg group ($n = 8$); and 3) T β 4-30 mg/kg group ($n = 8$). Sham rats underwent surgery without injury ($n = 6$). Our previous studies show that 6–8 animals per group provide sufficient power to reach significance after TBI induced by CCI.^{33,36,52,79} T β 4 used in the present study is a synthetic copy of the naturally-occurring 43-amino acid peptide provided by RegeneRx (Bethesda, MD). Immediately before injection, T β 4 in lyophilized powder form was weighed and dissolved in saline at a concentration of either 0.6 or 3 mg/ml. The animals received a dose of either 6 or 30 mg/kg with different injection volumes based on body weight. T β 4 dissolved in saline (RegeneRx, Bethesda, MD) was administered intraperitoneally starting at 6 hours after injury and then repeated one time daily once at 24 and 48 hours. The low dose of T β 4 (6mg/kg) was selected based on our previous studies.^{42,77,82} A higher dose group (30 mg/kg) was selected based on a recent T β 4 clinical trial in healthy humans,⁵⁸ with a dose translation from humans using the body surface area normalization method.⁵³ Animals in the saline-treated group received an equal volume of saline and were treated using the same protocol as T β 4. To detect proliferating cells, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected intraperitoneally into rats once daily for 10 days, starting 1 day after TBI. All rats were sacrificed at 35 days after TBI or surgery.

Morris Water Maze Test

To detect spatial learning impairments, a recent version of the Morris water maze (MWM) test was used¹⁰ and all animals were tested 5 days (from 31–35 days after TBI or surgery) before sacrifice. The swimming pool was located in a large room, where there were many clues external to the maze (pictures on the walls, lamps and a camera on the ceiling); these were visible from the pool and presumably used by the rats for spatial orientation. The position of the cues remained unchanged throughout the experiment. Data collection was automated by the HVS Image 2020 Plus Tracking System (US HVS Image, San Diego, CA). For data collection, the blue pool (1.8 m diameter) was subdivided into four equal quadrants formed by imaging lines. At the start of a trial, the rat was placed at one of four fixed starting points, randomly facing a wall (designated North, South, East and West) and allowed to swim for 90 seconds or until it found the platform. If the animal found the platform, it was allowed to remain on it for 10 seconds. If the animal failed to find the platform within 90 seconds, it was placed on the platform for 10 seconds. Throughout the test period the platform was located in the northeast (NE) quadrant 2 cm below water in a randomly changing position, including locations against the wall, toward the middle of the pool, or off-center but always within the target quadrant. If the animal was unable to find the platform within 90 seconds, the trial was terminated and a maximum score of 90 seconds was assigned. If the animal reached the platform within 90 seconds, the percentage of time traveled within the NE (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform and employed for statistical analysis. The advantage of this version of the water maze is that each trial takes on the key characteristics of a probe trial because the platform is not in a fixed location within the target quadrant.⁵⁹

Modified Neurological Severity Score (mNSS) Test

Neurological functional measurement was performed using the mNSS score test.⁷ The test was carried out on all rats preinjury and on Days 1, 4, 7, 14, 21, 28, and 35 after TBI. The mNSS is a composite of the motor (i.e., muscle status, abnormal movement), sensory (i.e., visual, tactile and proprioceptive), and reflex tests and has been employed in previous studies.³³ In this TBI model, injury in the left hemispheric cortex of rats causes sensory and motor functional deficiency with elevated scores on motor, sensory, and Beam Balance Tests in the early phase after injury (Day 1 after injury). Absent reflexes and abnormal movements can be measured in rats with severe injury. Slow recovery in asymmetry deficiency as reflected by Beam Balance Test results has been reported in unilateral brain injuries including TBI³³ and ischemia.⁷ This test is suitable for evaluating long-term neurological function after unilateral brain injury.

Tissue Preparation and Measurement of Lesion Volume

At day 35 after TBI, rats were anesthetized i.p. with chloral hydrate, then perfused transcardially first with saline solution, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Their brains were removed and post-fixed in 4% paraformaldehyde for 2 days at room temperature. The brain tissue was cut into 7 equally spaced 1-mm coronal blocks and processed for paraffin sectioning. A series of adjacent 6- μ m-thick sections were cut from each block in the coronal plane and stained with hematoxylin and eosin (H&E). For lesion volume measurement, the 7 brain sections were traced by a microcomputer imaging device (MCID) (Imaging Research, St. Catharines, Ontario, Canada), as previously described.⁸ The indirect lesion area was calculated (the intact area of the ipsilateral hemisphere is subtracted from the area of the contralateral hemisphere),⁷¹ and the lesion volume presented as a volume percentage of the lesion compared with the contralateral hemisphere. H&E sections from Blocks E and F containing hippocampus were used to acquire images of the dentate gyrus and CA3 regions at X 20 magnification. To evaluate the cell loss after TBI, we counted the cell number per millimeter in the dentate gyrus and CA3 regions.

Immunohistochemistry

To examine the effect of T β 4 on cell proliferation, coronal sections were histochemically stained with mouse anti-BrdU.³² Briefly, 6- μ m paraffin-embedded coronal sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling sections in 10-mM citrate buffer (pH 6.0) for 10 minutes.⁸ After washing with PBS, sections were incubated with 0.3 % H₂O₂ in PBS for 10 minutes, blocked with 1% BSA containing 0.3 % Triton-X 100 for 1 hour at room temperature, and incubated with mouse anti-BrdU (1:200; Dako, Carpinteria, CA) at 4°C overnight. After washing, sections were incubated with biotinylated anti-mouse antibody (1:200; Vector Laboratories, Inc., Burlingame, CA) for 30 minutes at room temperature. After an additional washing, sections were incubated with an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, Inc.). Diaminobenzidine (Sigma, St. Louis, MO) was then used as a sensitive chromogen for light microscopy. Sections were counterstained with hematoxylin. BrdU⁺ cells in the dentate gyrus and the cortex of ipsilateral hemispheres were examined at X20 magnification and counted.

Immunofluorescent Staining

To identify newly generated neurons in the dentate gyrus, a double labeling for BrdU and NeuN was performed. After dehydration, tissue sections were boiled in 10 mM citric acid buffer (pH 6) for 10 minutes. After washing with PBS, sections were incubated in 2.4 N HCl at 37°C for 20 minutes. Sections were then incubated with 1% BSA containing 0.3% Triton-X-100 in PBS, followed by incubation with mouse anti-NeuN antibody (1:200; Chemicon, Temecula, CA) at 4°C overnight. FITC-conjugated anti-mouse antibody (1:400; Jackson

ImmunoResearch, West Grove, PA) was added to sections at room temperature for 2 hours. Sections were then incubated with rat anti-BrdU antibody (1:200; Dako, Glostrup, Denmark) at 4°C overnight, followed by incubation with Cy3-conjugated anti-rat antibody (1:400; Jackson ImmunoResearch) at room temperature for 2 hours. Each of the steps was followed by 3 5-minutes rinses in PBS. Tissue sections were mounted with Vectashield mounting medium (Vector Laboratories). Images were collected with fluorescent microscopy and merged. NeuN/BrdU-colabeled cells in the dentate gyrus and the cortex were counted at a magnification of 40.⁸²

Cell Counting and Quantitation

The methods employed to count cells have been utilized in our previous studies.^{6,31,84} We did not use stereological counting methods but we counted cells using the principles of stereology.⁸⁴ This method permits a meaningful comparison of differences among groups. For quantitative measurements of BrdU⁺, NeuN⁺, and NeuN⁺/BrdU⁺ cells, 5 slides from each brain were analyzed, with each slide containing 5 fields of view in the lesion boundary zone from the epicenter of the injury cavity (bregma -3.3 mm), 3 fields of view in the ipsilateral CA3 and 9 fields of view in the ipsilateral dentate gyrus in the same section. The fields were digitized under the light microscope (Nikon, Eclipse 80i, Melville, NY) at either X 200 or X 400 magnification using a CoolSNAP color camera (Photometrics, Tucson, AZ) interfaced with MetaMorph image analysis system (Molecular Devices, Downingtown, PA). The immunopositive cells were calculated and divided by the measured areas, and presented as numbers per mm². Cell counts were performed by observers blinded to the individual treatment status of the animals. All counting was performed on a computer monitor to improve visualization and in one focal plane to avoid oversampling.⁸³ To evaluate whether Tβ4 administered i.p. reduces neuronal damage after TBI, the number of cells was counted in the dentate gyrus and CA3 region. Although H&E staining is not neuron-specific, the morphological characteristics of neuronal cells in the dentate gyrus and CA3 region aid in counting them. Counts were averaged and normalized by measuring the linear distance (in mm) of the dentate gyrus and CA3 for each section. For cell proliferation, the total number of BrdU⁺ cells was counted in the lesion boundary zone and dentate gyrus. The cells with BrdU (brown stained) that clearly localized to the nucleus (hematoxylin stained) were counted as BrdU⁺ cells. For analysis of neurogenesis, additional sections used in the above studies were used to evaluate neurogenesis in the dentate gyrus by calculating the density of BrdU-labeled cells and BrdU/NeuN-colabeled cells. We mainly focused on the ipsilateral dentate gyrus and its subregions, including the subgranular zone, granular cell layer, and the molecular layer. The number of BrdU⁺ cells (red stained) and NeuN/BrdU-colabeled cells (yellow after merge) were counted in the dentate gyrus and the lesion boundary zone. The percentage of NeuN/BrdU-colabeled cells over the total number of BrdU⁺ cells in the corresponding regions (dentate gyrus or cortex) was estimated and used as a parameter to evaluate neurogenesis.⁷⁵

Statistical Analyses

All data are presented as the mean ± standard deviation. Data were analyzed by analysis of variance (ANOVA) for repeated measurements of functional tests (spatial performance and sensorimotor function). For lesion volume, cell loss, cell proliferation and neurogenesis, a one-way ANOVA followed by post hoc Student-Newman-Keuls tests was used to compare the difference between the Tβ4-treated, saline-treated and sham groups. Correlation analysis of functional recovery with the lesion volume and cell counts was conducted by the Pearson linear correlation coefficient. Statistical significance was set at $p < 0.05$.

Results

Tβ4 Treatment Initiated 6 H Post-Injury Improves Spatial Learning

The modified version of MWM protocol was used to detect spatial learning deficits.^{10,44} The time spent in the correct quadrant (i.e., NE) by sham rats increased significantly from days 32–35 after surgery, as compared to time spent in the correct quadrant at day 31 (Fig. 1A, $F_{5,35} = 224.55$; $p < 0.001$). In testing of spatial memory, a statistically significant between-group effect on the time spent in the correct quadrant was noted in the MWM test (at Day 32, $F_{3,27} = 4.42$, $p = 0.012$; at Day 33, $F_{3,27} = 12.07$, $p = 0.004$; at Day 34, $F_{3,27} = 64.09$, $p < 0.001$; and at Day 35, $F_{3,27} = 20.88$, $p < 0.001$). Relative to the saline group, post-hoc testing demonstrated significantly increased time spent in the correct quadrant in both Tβ4-6 mg/kg group and Tβ4-30 mg/kg group at Day 33 ($p < 0.001$), Day 34 ($p < 0.001$) and Day 35 ($p < 0.001$). The Tβ4-30 mg/kg group significantly differed in time spent in the correct quadrant from the Tβ4-6 mg/kg group at Day 33 ($p < 0.001$), Day 34 ($p < 0.001$) and Day 35 ($p = 0.024$).

Tβ4 Treatment Initiated 6 H Post-Injury Reduces Neurological Severity Score

Figure 1B shows that there is no significant difference in the mNSS score between the saline- and Tβ4-treated groups at day 1 post TBI. However, compared to the saline-treated group, significantly improved scores were measured at Days 4–35 after TBI in the Tβ4-treated group (for Tβ4-6 mg/kg group at Days 4, 7, 14, 21, 28, and 35, with p values of < 0.001 , $= 0.013$, < 0.001 , < 0.001 , $= 0.024$ and $= 0.003$, respectively; and Tβ4-30 mg/kg group at Days 4–35, all p values < 0.001). Compared to the Tβ4-6 mg/kg group, the Tβ4-30 mg/kg group showed significantly reduced mNSS scores at Days 7, 14, 21, 28 and Day 35 with p values of < 0.001 , < 0.001 , < 0.001 , < 0.001 and $= 0.018$, respectively.

Tβ4 Treatment Initiated 6 Hours Post-Injury Reduces Cortical Lesion Volume in TBI Rats

Rats were sacrificed at 35 days post TBI for histological measurements. In the evaluation of contusion volume (Fig. 2), a significant between-group difference was found ($F_{2,21} = 27.95$; $p < 0.001$). As expected, sham-injured animals did not develop contusions. Tβ4 treatment (6 hours post-injury) at 6 mg/kg and 30 mg/kg reduced cortical lesion volume compared to the saline-treated group ($p < 0.001$ for the 6 and 30 mg/kg groups). The Tβ4-30 mg/kg group exhibited a significantly reduced lesion volume compared to the Tβ4-6 mg/kg group ($p = 0.013$).

Tβ4 Treatment Initiated 6 Hours Post-Injury Reduces Hippocampal Cell Loss in TBI Rats

When examined at 35 days post TBI (Fig. 3), the analysis of selectively vulnerable hippocampal regions adjacent to the contusion revealed significant between-group effects in both the dentate gyrus ($F_{3,27} = 82.43$, $p < 0.001$) and CA3 ($F_{3,27} = 26.91$; $p < 0.001$) regions. The neuron counts in the ipsilateral CA3 ($p < 0.001$) and dentate gyrus ($p < 0.001$) had significantly decreased after TBI (Fig. 3B and 3) compared to sham controls (Fig. 3A and D). Tβ4 treatment (6-hours post-injury) at 6 mg/kg and 30 mg/kg significantly reduced hippocampal cell loss in the CA3 region and dentate gyrus compared to saline-treated group ($p < 0.001$ for the 6 and 30 mg/kg groups). A significantly reduced cell loss was observed in the Tβ4-30 mg/kg group in the dentate gyrus ($p = 0.007$) and CA3 ($p = 0.005$) regions compared to the Tβ4-6 mg/kg group.

Tβ4 Treatment Increases Cell Proliferation in the Injured Cortex and Hippocampus

BrdU, an analog of thymidine, can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle, substituting for thymidine during DNA replication. BrdU staining is commonly used to detect proliferating cells. A statistically

significant between-group effect on the number of BrdU-positive cells was noted in the dentate gyrus ($F_{3, 27} = 176.62$, $p < 0.001$) and cortex ($F_{3, 27} = 1018.75$, $p < 0.001$). The post-hoc testing showed that the number of BrdU⁺ cells found in the ipsilateral cortex ($p < 0.001$) and dentate gyrus ($p < 0.001$) was significantly increased at 35 days after TBI, compared with sham controls (Fig. 4). However, T β 4 treatment further increased the number of BrdU⁺ cells in the cortex ($p < 0.001$) and dentate gyrus ($p < 0.011$) after TBI compared to saline controls (Fig. 4). Significantly increased cell proliferation was noted in the T β 4-30 mg/kg group compared to the T β 4-6 mg/kg group ($p < 0.001$).

T β 4 Treatment Increases Neurogenesis in the Injured Dentate Gyrus

To identify newly generated neurons, double immunofluorescent staining for BrdU (proliferating marker) and NeuN (mature neuronal marker) was performed. A statistically significant between-group effect on the number of newborn neurons was noted in the dentate gyrus ($F_{3, 27} = 288.72$, $p < 0.001$). The number of NeuN/BrdU-colabeled cells (newborn neurons) was significantly greater in the injured dentate gyrus ($p < 0.001$) after TBI compared to sham controls. T β 4 treatment significantly increased the number of newborn neurons in the injured dentate gyrus ($p < 0.001$ for both doses of T β 4) compared to saline controls (Fig. 5), while the T β 4-30 mg/kg group exhibited a significantly increased number of newborn neurons compared to the T β 4-6 mg/kg group ($p < 0.001$). As compared to sham animals (as baseline), neurogenesis increased by 2.1-, 4.5-, and 5.6-fold for saline, T β 4-6 mg/kg, and T β 4-30 mg/kg groups, respectively.

Correlation of Functional Outcome with Lesion Volume, Cell Number, and Neurogenesis

To analyze the potential mechanism underlying the difference in functional outcome after T β 4 treatment, we quantified the correlation of sensorimotor function with cortical lesion volume (Fig. 6A), spatial learning with hippocampal cell counts (Fig. 6B) and with dentate gyrus neurogenesis (Fig. 6C) examined at Day 35 after injury. The sensorimotor functional deficits are highly and significantly correlated with the cortical lesion volume ($R^2 = 0.7781$, $p < 0.001$). The spatial learning is highly and significantly correlated with the cell number in the dentate gyrus ($R^2 = 0.7287$, $p < 0.001$) and CA3 region ($R^2 = 0.7285$, $p < 0.001$). In addition, the improved spatial learning is highly correlated with the number of newborn neurons in the dentate gyrus ($R^2 = 0.8104$, $p < 0.001$). These data suggest that the T β 4-improved functional recovery is due in part to its neuroprotection (reduced cortical lesion and hippocampal cell loss) and neurogenesis (increased newborn neurons) in the dentate gyrus.

Discussion

The present study provides the first evidence that T β 4 treatment initiated 6 hours post injury provides a neuroprotective effect on reducing cortical lesion volume in rats after TBI. T β 4 treatment significantly improves long-term behavioral benefits, including spatial learning and sensorimotor functional recovery, as evaluated by MWM and mNSS tests. The improvements in sensorimotor function are significantly correlated with the effect of T β 4 treatment on reducing cortical lesion volume and increasing cortical cell proliferation. Improved spatial learning is significantly correlated with the effect of T β 4 treatment on reducing hippocampal cell loss and increasing DG cell proliferation and neurogenesis. Our findings demonstrate that early T β 4 treatment followed by repeated dosing not only provides neuroprotection but also promotes neurogenesis, indicating that T β 4 is a potential therapy for TBI patients.

The findings in the present study differ from our previous study with delayed T β 4 treatment for TBI. Our previous study demonstrates that T β 4 treatment initiated 24 hours post-injury

did not reduce cortical lesion volume but improved long-term functional recovery,⁷⁷ which is consistent with our recent finding that Tβ4 treatment initiated 24 hours post-stroke improves functional recovery.⁴² The improved functional recovery may derive from the neurorestorative effects of delayed Tβ4 treatment including angiogenesis, oligodendrogenesis, and neurogenesis.^{43,77} Our present study shows that Tβ4 treatment initiated 6 hours post-injury significantly reduces cortical lesion volume and improves sensorimotor functional recovery (that is, reduced mNSS), clearly demonstrating that Tβ4 is neuroprotective and promising for treatment of TBI. Our current data are in line with the previous findings that neuroprotective agents significantly reduce brain damage including erythropoietin⁹ and cyclosporin A⁶⁷ when administered within 6 hours after TBI. In addition, our present data show that Tβ4 treatment initiated 6 hours post-TBI reduces hippocampal cell loss. Similar neuroprotective effect (reduced hippocampal cell loss) was also seen with Tβ4 treatment initiated 24 hours post-injury.⁷⁷ Our previous data demonstrate that apoptosis induced by CCI occurs as early as 2 hours after injury and persists up to 2 weeks afterwards.²⁷ This finding supports the hypothesis that the early therapy combined with late repeated dosing for treatment of TBI may be required to provide maximum efficacy. The early treatment provides neuroprotection and the repeated delayed treatment maintains neuroprotection and promotes neurorestorative effects including neurogenesis as demonstrated in the present study and our previous study.⁷⁷ Although we did not investigate apoptosis in the present study, Tβ4 provides the neuroprotection likely through its anti-apoptotic effect. Tβ4 reduces apoptosis in many cells including human corneal epithelial cells²⁴ and gingival fibroblasts,⁵⁴ rat cortical astrocytes⁸¹ and neurons.¹¹ Intracerebroventricular administration of Tβ4 significantly reduces hippocampal neuronal loss induced by kainic acid.⁵⁰ Several other Tβ4-derived peptides have been screened for biological activity.⁶⁴ The first 15 amino acids of Tβ4 have been found to be major sites for cytoprotection and for antiapoptotic activity.⁶⁴ In the present study, the rats were sacrificed at 35 days after TBI, which may not be suitable for evaluating the early antiapoptotic effect of Tβ4 treatment. Clearly, a more in-depth study evaluating the antiapoptotic effect of Tβ4 treatment with animals sacrificed at early and different time points after TBI is warranted. In addition, whether a single dose of Tβ4 treatment initiated 6 hours after TBI can afford a similar effect on neuroprotection and neurogenesis needs further investigation.

Neurogenesis occurs in the subventricular and subgranular zones of mammalian adult brains.^{20,85} TBI alone enhances neurogenesis in the dentate gyrus and subventricular zone.^{34,55,68,73,76} Newborn neurons in the dentate gyrus after TBI are capable of projecting axons into the CA3 region and may integrate in the existing neuronal circuitry.^{16,69} Injury-induced neurogenesis may partially contribute to spontaneous functional recovery after TBI. However, this endogenous neurogenesis is limited in the adult brain after injury. Previous studies from us and others demonstrate that increasing neurogenesis by therapeutic treatments may contribute to improved functional recovery after TBI.^{35,39,46,55,68,69,78} In the present study, although the causal link was not investigated, our correlation data indicate that the improvement in spatial learning is highly correlated with increased neurogenesis and cell counts at day 35 after TBI. Electrophysiological maturation of newborn neurons in the dentate gyrus progresses over a period of 2–7 weeks after cell division in rodents, which is accompanied by changes in expression of the immature neuron markers for the first 3 weeks, with a subsequent transition to the mature neuronal marker NeuN by 4–6 weeks.^{29,86} In the present study, Tβ4 treatment further increases neurogenesis in the dentate gyrus after TBI compared to saline treatment. Neurogenesis plays a critical role in spatial learning and memory.^{28,35,39,69} In rodents, it is well established that the newborn neurons are involved in olfaction as well as in certain forms of memory and learning under normal and disease conditions.⁶⁰ Eriksson et al. published the first pivotal study on hippocampal neural progenitor cells and neurogenesis in the human brain.¹⁷ Neurogenesis is involved in processes such as learning, memory, and depression, and may also be involved in

regenerative attempts after injuries to the central nervous system while the contribution of neurogenesis to these phenomena remains to be elucidated, especially in humans.⁴⁰ Collectively, our data suggest that T β 4-induced increased neurogenesis of the dentate gyrus and reduced neuronal loss of the CA3 region and dentate gyrus may contribute to improvement in spatial learning after T β 4 treatment.

Our present study indicates that the higher dose of T β 4 (30 mg/kg) provides better functional and histological outcomes compared to the lower dose (6 mg/kg). A pharmacokinetic study of T β 4 demonstrates that peak concentration is found in the brain at 40 minutes following intraperitoneal administration of T β 4 in mice.⁴¹ In addition, the intravenous administration of T β 4 over a dose range of 0.6–60 mg/kg is safe and tolerated without evidence of dose-limiting toxicity in all study animals.¹² A recent clinical trial shows that intravenous administration of T β 4 as a single dose or in multiple daily doses for 14 days over a dose range of 42–1260 mg is well tolerated with no evidence of dose-limiting toxicity in healthy humans.⁵⁸ The mean half-life of T β 4 in plasma increased with a single intravenous dose from 0.95 hours at 42 mg/subject, to 1.2 hours at 140 mg/subject, to 1.9 hours at 420 mg/subject, and to 2.1 hours at 1260 mg/subject.⁵⁸ Whether the higher dose of T β 4 (30 mg/kg) used in the present study is optimal remains unknown. This rat dose is equivalent to a human equivalent dose of 4.86 mg/kg, assuming 60 kg human, with a dose translation from rats using the body surface area normalization method.⁵³ Our recent studies demonstrate that intraperitoneal injection of T β 4 at 6 mg/kg starting at 24 hours after brain injury every 3 days improves neurological function in rat models of embolic stroke and TBI.^{42,77} To date, there is no effective pharmacological therapy available to promote functional recovery after TBI.^{30,45,57} Inflammation, oxidative injury, excitotoxicity, apoptosis, and hypoxia/ischemia contribute to secondary brain injury following TBI.^{72,74} Clinical TBI trials targeting a single pathophysiological pathway have failed; a successful therapy may require targeting multiple injury pathways.^{45,72} T β 4 protects human corneal epithelial cells against oxidative injury,²⁵ induces hair growth via stem cell migration and differentiation,⁴⁹ promotes epicardium-derived neovascularization in the adult heart,^{56,62} reduces rat hippocampal neuronal loss by kainic acid excitotoxicity,⁵⁰ reduces apoptotic death of chick and rat neurons,¹¹ and inhibits inflammation.⁶⁵ These studies provide convincing data that T β 4 is a regenerative multifunctional peptide (it has anti-inflammatory, anti-oxidative, anti-apoptotic, pro-angiogenic, and tissue-protective effects), which could be a safe and promising agent for treatment of TBI.

T β 4 is mainly recognized as a regulator of actin polymerization by its ability to form a complex with G-actin.⁶⁴ This peptide has been implicated in angiogenesis, apoptosis, blood coagulation, carcinogenesis, lymphocyte maturation, and wound healing.^{26,70} T β 4 is also involved in lesion-induced neuroplasticity through upregulation of microglia.^{15,47} The signaling pathways beyond actin sequestration remain largely unknown. T β 4 lacks stable folded structure in aqueous solution and acquires specific folded structures only on binding to a partner protein; T β 4 offers many possibilities for interaction with multiple partners.^{4,13,64} A candidate extracellular receptor of high affinity for T β 4 is the β subunit of cell surface-located ATP synthase, which would allow extracellular T β 4 to signal via a purinergic receptor to promote endothelial cell migration.¹⁹ T β 4 induces endothelial progenitor cell migration via the PI3K/Akt/eNOS signal transduction pathway.⁵¹ Internalization of exogenous T β 4 may be responsible for the antiapoptotic effects of exogenous T β 4 on human corneal epithelial cells.²⁴ Internalization of the exogenous T β 4 followed by activation of integrin-linked kinase and Akt seem to be the major mechanisms that promote the survival of cardiomyocytes.^{3,21} The Ku80 subunit of ATP-dependent DNA helicase II is associated with T β 4 and functions as a novel receptor for T β 4, mediating its intracellular activity.² Ku80 has been implicated in multiple nuclear processes, e.g., DNA repair, telomere maintenance and apoptosis.²² Whether these signaling pathways mediate

neuroprotection and neurorestoration of T β 4 is unclear. Further investigation on these aspects is required.

In conclusion, the therapeutic efficacy of early T β 4 treatment for TBI demonstrated in the present study suggests that T β 4 is a potential therapy for TBI patients. Further investigation of the optimal dose and therapeutic window of T β 4 treatment for TBI and the associated underlying mechanisms is warranted.

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Fig. 1.

The effect of Tβ4 on functional recovery after TBI. **A:** Effect of Tβ4 on spatial learning function 31–35 days after TBI. TBI significantly impairs spatial learning at days 32–35 compared to sham controls ($p < 0.05$). Tβ4 improves spatial learning performance (i.e., increased percentage of time spent in the correct quadrant of the Morris water maze) measured by a modified version of the Morris water maze test at days 33–35 compared with the saline group ($p < 0.05$). **B:** Effect of Tβ4 on sensorimotor function detected on the modified neurological severity scores (mNSS). Tβ4 treatment significantly lowers mNSS scores at days 4–35 compared to the saline group ($p < 0.05$). Data represent mean $\pm$ SD. * $p < 0.05$ vs saline group. # $p < 0.05$ vs Tβ4-6 mg/kg group. n (rats/group) = 6 (sham), 7 (saline), 8 (Tβ4-6 mg/kg), and 8 (Tβ4-30 mg/kg).

Fig. 2.

The effect of Tβ4 on cortical lesion volume examined 35 days after TBI. TBI caused significant cortical tissue loss. Tβ4 treatment significantly reduced lesion volume caused by TBI compared to the saline-treated groups ($p < 0.05$). Scale bar = 3 mm. Data represent mean $\pm$ SD. * $p < 0.05$ vs saline group. # $p < 0.05$ vs Tβ4-6 mg/kg group. n (rats/group) = 7 (saline), 8 (Tβ4-6 mg/kg), and 8 (Tβ4-30 mg/kg).

Fig. 3.

The effect of Tβ4 on cell number in the ipsilateral dentate gyrus and CA3 region at 35 days after TBI. H&E staining: a–h. Treatment with Tβ4 (c, d, g, h) significantly reduces cell loss as compared with the saline-treated group (b, f) ($p < 0.05$). The cell number in the dentate gyrus and CA3 region is shown in (i). Scale bar = 50 μm (h, applicable to a–h). Data represent mean $\pm$ SD. * $p < 0.05$ vs saline group. # $p < 0.05$ vs Tβ4-6 mg/kg group. n (rats/group) = 6 (sham), 7 (saline), 8 (Tβ4-6 mg/kg), and 8 (Tβ4-30 mg/kg).

Fig. 4.

The effect of Tβ4 on cell proliferation in the injured cortex and dentate gyrus 35 days after TBI. TBI alone (b, f) significantly increases the number of BrdU⁺ cells in the ipsilateral cortex and dentate gyrus compared to sham controls (a, e) ($p < 0.05$). Tβ4 treatment significantly increases the number of BrdU⁺ cells in these regions (c, d, g, h; arrow as examples showing BrdU⁺ cell) compared to the saline-treated groups ($p < 0.05$). The number of BrdU⁺ cells is shown in (i). Scale bar = 25 μm (h, applicable to a–h). Data represent mean ± SD. * $p < 0.05$ vs saline group. # $p < 0.05$ vs Tβ4-6 mg/kg group. n (rats/group) = 6 (sham), 7 (saline), 8 (Tβ4-6 mg/kg), and 8 (Tβ4-30 mg/kg).

Fig. 5.

Double immunofluorescent staining for BrdU (red) and NeuN (green) to identify newborn neurons (yellow after merge) in the brain (a–d). The number of NeuN⁺ cells colabeled with BrdU is shown in (e). Scale bar = 25 μm (d, applicable to a–d). Data represent mean ± SD. *p < 0.05 vs saline group. #p < 0.05 vs Tβ4-6 mg/kg group. n (rats/group) = 6 (sham), 7 (saline), 8 (Tβ4-6 mg/kg), and 8 (Tβ4-30/kg).

Fig. 6.

Correlation of functional recovery with lesion volume, cell loss, and cell proliferation and neurogenesis examined 35 days after TBI. A: Significant correlation of mNSS with lesion volume. $p < 0.001$. B: Significant correlation of spatial learning with cell loss in the DG and CA3 region. $p < 0.001$. C: Significant correlation of spatial learning with BrdU+ cells in the cortex. D: Significant correlation of mNSS with BrdU+ cells in the DG. $p < 0.001$. E: Significant correlation of spatial learning with newborn neurons in the DG. n (rats/group) = 6 (sham), 7 (saline), 8 (T β 4-6 mg/kg), and 8 (T β 4-30 mg/kg).