Published in final edited form as:

Neurosci Lett. 2017 October 17; 659: 7–13. doi:10.1016/j.neulet.2017.08.064.

Thymosin β 4 for the Treatment of Acute Stroke in Aged Rats

Daniel C Morris¹, Wing Lee Cheung¹, Richard Loi¹, Talan Zhang³, Mei Lu³, Zheng G Zhang², and Michael Chopp^{2,4}

¹Department of Emergency Medicine, Henry Ford Health Systems, Detroit, MI 48202

²Department of Neurology, Henry Ford Health Systems, Detroit, MI 48202

³Department of Public Health Sciences, Henry Ford Health Systems, Detroit, MI 48202

⁴Department of Physics, Oakland University, Rochester, MI 48309

Abstract

Thymosin β 4 (T β 4) is a 5K peptide which influences cellular migration by inhibiting organization of the actin-cytoskeleton. T β 4 has neurorestorative properties and is a potential candidate for the treatment of sub-acute stroke. Previous research demonstrated that T β 4 improved neurological outcome in a young (3 months) rat model of embolic stroke. We hypothesized that T β 4 would improve neurological outcome in an aged rat model of embolic stroke when administered 24 hours after embolic stroke. Aged Male Wistar rats (Charles River, France 18–21 months) were subjected to embolic middle cerebral artery occlusion (MCAo). Rats were randomized to receive T β 4 (12 mg/kg, RegeneRx Biopharmaceuticals, Inc.) or control 24 hrs after MCAo and then every 3 days for 4 additional doses. The dose of 12 mg/kg was the maximal dose of T β 4 that showed functional improvement in a young rat model of embolic stroke. Functional tests (adhesive-removal test (ART), foot fault test (FFT) and the modified Neurological Severity Score (mNSS)) were performed weekly. The rats were sacrificed 56 days after MCAo and lesion volumes were measured. Immunohistochemical analysis for oligodendrogenesis, myelination and gliosis was also performed. Twenty-three rats were included in the study: control group (n=12) and T β 4 group (n=11). After randomization, there were three deaths in both the control and T β 4 groups. The T β 4 treatment reduced infarct volume by more than 50% ($12.8\% \pm 9.3\%$, mean $\pm$ SE, $p < 0.05$) compared to the control group ($26.0\% \pm 4.3\%$). However, T β 4 did not show improvement in functional outcome compared to control. There was no significant increase in oligodendrogenesis, myelination and gliosis between control and treatment with T β 4, however, we unexpectedly observed that overall (control and T β 4 groups) astrocytic gliosis as measured by GFAP immunoreactivity was significantly inversely correlated with neurological outcome measured using the modified Neurological Severity Score (mNSS) ($p < 0.01$), suggesting that greater gliosis may be related to improvement of neurological outcome in aged rats. In summary, T β 4 treatment of stroke aged rats significantly reduces infarct volume compared to vehicle treated stroke,

To whom correspondence should be addressed: Daniel C. Morris, Department of Emergency Medicine, 2799 West Grand Blvd, CFP-2, Detroit, MI, USA, Tel: (313)-916-0243; Fax: (313)-916-1318; dmmorris4@hfhs.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

however, T β 4 treatment did not show improvement in functional outcome, myelination or gliosis when compared to control. GFAP staining was significantly inversely correlated to improvement in the mNSS, suggesting that gliosis in the aged rat may be of benefit in improvement of functional outcome.

Keywords

Thymosin β 4; neurorestorative; stroke; gliosis

Introduction

Age is a well-known risk factor for stroke with 75% of all strokes occurring in people over the age of 65 years old [1]. The risk of having a stroke more than doubles each decade after the age of 55 years old. Furthermore, recovery from stroke is also influenced by age; youth fare better after stroke than the aged given the same degree of ischemic insult [2]. In experimental models of stroke, aged rats recover more slowly, less completely and have higher mortality rates when compared to younger rats [2, 3]. Based on these facts, the aged post-acute stroke rat is the most clinically relevant model to investigate therapies hypothesized to improve stroke outcome.

Neurorestorative agents treat the surrounding intact healthy neural tissue and promote repair of damaged neural tissues from neurological injury [4]. Neurorestorative agents act on intact parenchymal cells including neuroprogenitor (adult neural stem cells), oligodendrocyte progenitor cells, astroglial cells, and cerebral endothelial cells, to promote neurogenesis, oligodendrogenesis, axonal sprouting, synaptogenesis and angiogenesis, in the injured brain. These restorative processes are associated with improvement in neurological functional outcome [5].

Thymosin β 4 (T β 4) is a developmentally expressed 43-amino acid peptide that sequesters G-actin monomers [6–8]. Based on our observations that T β 4 improves recovery in four different models of neurological injury: stroke, multiple sclerosis, traumatic brain injury and peripheral neuropathy, we proposed that T β 4 is a neurorestorative agent [9–12]. A common observation in all four models is oligodendrogenesis and/or the production of mature myelin-secreting oligodendrocytes (OL) from oligoprogenitor (OPC) cells, or in the case of peripheral neuropathy, production of myelin from Schwann cells. In order to develop T β 4 for use in clinical trials, we performed a dose-response study in our rat model of embolic stroke in young rats and determined that a T β 4 dose of 2 and 12 mg/kg improved neurological outcome [13]. Specific observations showed that T β 4 increased proliferating OPCs in the subventricular zone (SVZ) and in the white matter of the corpus callosum, as well as increased myelination in the ischemic vulnerable striatal boundary in the 12 mg/kg treated group.

The next logical step for clinical translation was to test T β 4 in an aged rat model of embolic stroke. We hypothesized that T β 4 would show functional improvement in aged Wistar rats (Charles River) treated with 12 mg/kg T β 4, the maximal dose that showed improvement in young rats. However, our results differed from those obtained in the young rat model of

embolic stroke, suggesting an aged related effect on the treatment of acute embolic stroke with T β 4.

Material and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Aged (18–20 months) male Wistar rats imported from France were obtained from Charles River Laboratories. These aged rats (n=29) were acquired at age 15 months and kept at Henry Ford Health Systems animal care facilities for 3 months before initiation of the study. This group of rats was used to test the therapeutic efficacy of T β 4 at 12 mg/kg, the highest dose that showed efficacy in the dose-response study using young rats.

Embolic stroke rat model

The middle cerebral artery (MCA) of the aged male Wistar rats was occluded by placement of an embolus at the origin of the MCA, as described in previous publications [13, 14]. Briefly, under an operating microscope (Carl Zeiss, Inc., Thornwood, NY, USA), the right common carotid artery (CCA), the right external carotid artery (ECA) and the internal carotid artery (ICA) were isolated via a 3 cm ventral neck midline incision. A 6–0 silk suture was loosely tied at the origin of the ECA and ligated at the distal end of the ECA. The right CCA and ICA were temporarily clamped using a curved microvascular clip (Codman & Shurtleff, Inc., Randolph, MA, USA). A modified PE-50 catheter filled with a fibrin rich clot from a donor rat, was attached to a 100- μ l Hamilton syringe, and introduced into the ECA lumen through a small puncture. The suture around the origin of the ECA was tightened around the intraluminal catheter to prevent bleeding, and the microvascular clip removed. The catheter was then gently advanced from the ECA into the lumen of the ICA to reach the origin of the MCA. The clot along with 5 μ l of saline in the catheter was injected into the ICA over 10 seconds. The catheter was withdrawn from the right ECA 5 min after injection and ligated. Buprenex was administered intraperitoneally (IP) post-surgery at a dose of 0.01 mg/kg if the rat showed signs of distress.

Experimental Design

T β 4 (RegeneRx Biopharmaceuticals Inc) was administered IP 24 hrs after MCA occlusion (MCAO) and then every 3 days for 4 additional doses. Randomization was performed using a computer generated list. Since this study was designed to observe functional outcome, we only included those rats which completed the functional tests until time of sacrifice (56 days). For labeling proliferating cells, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected IP into rats 24 hours after MCAo and then daily for 7 days. Fifty-six days after MCAO, rats were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and transcardially perfused with saline followed by 4% paraformaldehyde. The rat was then decapitated and brain removed and fixed in 4% paraformaldehyde.

Neurological Functional tests

In the embolic stroke rat model, a battery of behavioral tests was performed at 1, 7, 14, 21, 28, 35, 42, 49 and 56 days after MCAO by an investigator who was blinded to the

experimental groups. The battery of tests consisted of the adhesive-removal test (ART), the modified Neurological Severity Score (mNSS) and foot fault test (FFT) [15–17]. Briefly, the ART measures both motor and sensory function and it involves placing two small pieces of adhesive-backed paper dots on the wrist of each forelimb (of equal size, 113.1 mm²) to act as bilateral tactile stimuli occupying the distal-radial region. The time required for the rat to remove both stimuli from each limb was recorded in 5 trials per day. Each animal received 5 trials on all testing days after MCA occlusion and the mean time required to remove both stimuli from limbs was recorded. In order to increase sensitivity of the test, the adhesive-backed paper dots were reduced in size by one-half at day 35. The mNSS test is a composite score in which motor, sensory, balance and reflex measures are used to calculate a value ranging from 1 to 18, with the higher score implying greater neurological injury. Points are awarded for the inability to perform the tasks or for the lack of a tested reflex (normal score 1, maximal deficit score 18). Rats with a mNSS less than 8 were excluded from the study. The FFT tests cerebellum function by measuring the ability of the rat to walk along an elevated hexagonal wire grid and the number of times the paw slips (foot fault) between the wires. The total number of foot faults is divided by the total number of steps the rat uses to cross the grid. The average number of foot faults of a normal rat is 2–3%. As anticipated, rats which died due to the MCAo procedure (at day 1 or 2 before and after randomization) were excluded from the analysis.

Histological and immunohistochemical assessment

After fixed in paraformaldehyde, the brains were embedded in paraffin and cut into seven equally spaced (2 mm) coronal blocks. A series of adjacent 6 µm-thick sections was cut from each block in the coronal plane and was stained with hematoxylin and eosin. Seven brain sections were traced using a microcomputer imaging device (MCID) image analysis system (Imaging Research, St. Catharines, Canada) by laboratory associates who were blinded to the dose and functional testing results. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Lesion volume is represented as a volume percentage of the lesion compared with the contralateral hemisphere.

Standard paraffin blocks were obtained from the center of the lesion, corresponding to coronal coordinates for bregma –1–1 mm. A series of 6 µm thick sections at various levels (100 µm interval) were cut. Immunostaining was performed on these sections. Antibodies used for identification of OPCs and OLs were NG-2 (chondroitin sulfate proteoglycan) (1:800, polyclonal rabbit, incubated overnight at 4°C, Chemicon, CA) and APC (CC1) (adenomatous polyposis coli) (1:20, monoclonal mouse, incubated 60 minutes at room temperature, Genway, CA), respectively. To identify proliferating OPCs and OLs, double immunostaining of NG2/BrdU and APC/BrdU was performed, respectively. Antibodies used for identification of Aquaporin4 (AQ4) and endothelial cells were AQ4 (1:1500, polyclonal rabbit, incubated overnight at 4°C, Millipore, Germany) and Endothelium Barrier antigen (EBA) (1:1000, monoclonal mouse, incubated overnight at 4°C, Covance, NJ), respectively. Double immunolabeling was visualized by secondary antibodies conjugated to FITC and Cy3 (Jackson Immuno).

Myelin basic protein (MBP) antibody (1:1000, monoclonal mouse, incubated 60 minutes at room temperature, Covance, CA) was used to identify myelinated axons. Glial fibrillary acidic protein (GFAP) antibody (1:1000, polyclonal rabbit, incubated overnight at 4C, Dako, Denmark) was used to identify reactive gliosis. Following sequential incubation with biotin-conjugated anti mouse or rabbit IgG (dilution 1:200, Vector laboratories, INC), the sections were treated with an ABC kit (Vector laboratories, INC). Diaminobenzidine was then used as a sensitive chromogen for light microscopy. Isotype matched non-immune IgG controls were performed for each antibody.

Image acquisition and quantification

Coronal sections were digitized using a 40× objective using a three-CCD color video camera (Sony DXC-970MD) interfaced with a MCID system. Laboratory associates performing the quantitative analysis were blinded to the dose and functional testing results. For quantitative analysis of proliferating OPCs, the numbers of double labeled NG2 and BrdU immunoreactive cells were counted throughout the ipsilateral SVZ (ranging from 13–17 fields of view), ischemic boundary zone (IBZ) (8 fields of view) and the corpus callosum (CC) (4 fields of view). The number of positive cells within three coronal sections per rat was averaged to obtain a mean number of cells. The total measured area of the field of views were calculated. A similar analysis was performed using double labeling of APC and BrdU cells. AQ4 and EBA were analyzed using a computer program to count and auto-threshold double labeled immunoreactivity throughout the IBZ of the cortical area. Data are presented as the density of immunoreactive cells (cells/mm²) for NG2 and APC. For AQ4/EBA, the average percentage of increase or decrease is shown.

For quantification of myelinated axons, MBP immunoreactive area within the IBZ and CC and the contralateral homologous area were digitized. Three coronal sections (100µm interval) from the center of the ischemic core (bregma 0.2 mm) per animal in each hemisphere were analyzed. The fields of view of IBZ and CC of each section were analyzed. Comparing the measured area of the field of view within the ischemic hemisphere to its corresponding homologous area in the contralateral hemisphere, the average percentage of increase or decrease of MBP or AQ4 and EBA reactivity within both the IBZ and/or CC regions of each section is presented. In addition, reactive gliosis was quantified by measurement of GFAP immunoreactivity in eight fields of view within the IBZ. Data are presented as the percentage of increase in area at the IBZ compared with the contralateral homologous region on the same section.

Data Analysis and Statistical Procedures

Data transformations were considered if data were not normal. As a result, the ranked ART, FFT and mNSS were used for the comparison, because the functional data were not normally distributed. To mimic analysis employed in clinical stroke recovery studies [18], a global test using generalized estimation equations (GEEs), PROC GENMOD in SAS [19], was performed to evaluate the effect of Tβ4 on functional recovery measured from three neurological behavior tests at day 56, as the primary outcome compared to controls. The GEE approach was also used to study the Tβ4 effect on the functional recovery at the other time points (secondary endpoints), as well as the histological evaluation (remyelination, the

number of proliferating OPCs, and mature OLs) and lesion volume measured at day 56, as exploratory endpoints. Multilevel generalized linear mixed models were fit using proc GLIMMIX to test the treatment effect of AQ4/EBA or GFAP immunoreactivity in each section. Correlation of GFAP immunoreactivity was calculated between infarct volume, mNSS, adhesive removal test and foot fault test.

Results

The study consisted of administration of T β 4 at 12 mg/kg to aged (18–20 months) Wistar rats. A total of 29 rats had MCAO surgery performed; however, six rats died before randomization. Twenty-three rats were randomized into the study, 17 survived to the final endpoint; three died in the control group (two rats died within 24 hours of randomization and one rat died three days after randomization) and three died in the treated (one rat died within 24 hours of randomization and two rats died within 48 hours of randomization) group before the study ended.

Neurological functional outcome and lesion volume

To determine if T β 4 dose-dependently improves functional outcome after stroke, we performed behavioral tests one day after MCAO and then weekly for 56 days. The results showed no functional improvement overall, or on final day 56, ART ($41.56 \text{ sec} \pm 10.10$ vs $35.23 \text{ sec} \pm 12.55$), mNSS (4.89 ± 0.57 vs 4.00 ± 1.22) and FFT (6.00 ± 1.09 vs 6.56 ± 3.47) (control vs treatment, mean $\pm$ SE), $p>0.05$, (Figure 1). However, a significant difference in lesion volumes was observed, $26.04 \pm 4.29\%$ and $12.77\% \pm 3.61\%$, (mean $\pm$ SE) (control vs treatment, $\pm$ SE), $p<0.05$, (Figure 2). Because of these significant differences, coronal sections were analyzed to determine if T β 4 treatment promotes OPC and OL proliferation, myelination and gliosis after stroke in this aged rat model of embolic stroke.

T β 4 does not increase markers of myelination in aged rats

To determine the effect of T β 4 treatment on OPCs and OLs after stroke, densities of NG2+/BrdU+ (OPCs) and APC+/BrdU+ (OLs) were measured on double immunofluorescently stained brain sections. OPC density (cells/mm²) in the SVZ (13.61 ± 9.76 vs 12.83 ± 11.95), IBZ (2.97 ± 2.36 vs 2.79 ± 4.13) and CC (1.04 ± 1.66 vs 3.94 ± 4.73), (control vs treatment) showed no differences when compared to the control ($p>0.05$) (mean $\pm$ std). Likewise, OL density (cells/mm²) in the SVZ (3.88 ± 4.42 vs 6.91 ± 8.41), IBZ (1.53 ± 4.33 vs 1.91 ± 2.47) and CC (2.73 ± 3.13 vs 1.12 ± 2.06), (control vs treatment) showed no differences when compared to the control ($p>0.05$) (mean $\pm$ std) (Figure 3 and 4). MBP reactivity showed no differences (control vs treated) in the IBZ ($91.0 \pm 13.0\%$ vs $94.0 \pm 11.0\%$) or CC ($114.0 \pm 24.0\%$ vs $93.0 \pm 26.0\%$) ($p>0.05$) (mean $\pm$ std) (Figure 5).

T β 4 does not significantly alter reactive gliosis in aged rats

Ischemic damage after stroke results in reactive gliosis leading to formation of the glial scar [20–22]. GFAP staining along the ischemic boundary zone was measured. The ischemic side was compared to the contralateral (non-ischemic) side to generate a percentage of GFAP staining. T β 4 did not significantly alter reactive gliosis ($213 \pm 104\%$ vs $145 \pm 45\%$) (treatment vs control, mean $\pm$ std) ($p=0.09$) (Figure 6). However, GFAP immunoreactivity in

all aged rats (control and T β 4 groups) was significantly and inversely correlated (Pearson partial correlation coefficient = 0.67) ($p < 0.01$) with mNSS and marginally significantly correlated with the ART ($p = 0.077$), while the FFT showed no significant correlation. Collectively, these results suggest that aged rats with greater gliosis have better functional improvement with regard to motor and sensory function. Moreover, increased area of GFAP immunoreactivity was marginally inversely correlated with a reduction in infarction volume ($p = 0.067$).

T β 4 increases AQ4 and EBA cortical expression in aged rats

To determine the effect of T β 4 treatment on the reduction of infarct volume, blood brain barrier integrity and astrocytic response, we quantified the area of AQ4 and EBA double staining in cortical IBZ. Four rats in the control group and three rats in the T β 4 group had infarcts extending to the cortical area. AQ4/EBA reactivity was marginally significantly increased in the treated group ($0.06 \pm 0.04\%$ vs $0.11 \pm 0.010\%$, $p = 0.056$) (control vs treated) (mean $\pm$ std) (Figure 7). Moreover, T β 4 treatment attenuated the loss of AQ4 expression relative to the level of expression on the corresponding contralateral hemisphere, 0.058% vs 0.106% , $p = 0.056$ (control vs treated).

Discussion

Induction of recovery after stroke in the aged patient presents a treatment challenge [23]. Significant costs both in loss of quality of life and the societal burden in caring for aged stroke patients necessitate development of a treatment strategy. The failure of many stroke clinical trials testing novel agents may in part be attributed to the preclinical testing in young rodents and not in aged rodents [2]. Testing of candidate drugs for treatment of stroke in aged animals has been recommended by the Stroke Therapy Academic Industry Roundtable (STAIR) Committee and the Stroke Progress Review Group [24]. The results of this study demonstrate the differences in outcome that can occur when testing a therapeutic approach between the young and the aged. Our previously published work showed that in young rats, T β 4 (12 mg/kg) administered 24 hours after stroke demonstrated significant ($p < 0.05$) improvement in functional outcome, 33% in the ART, 26% in the mNSS and 35.5% in the FFT; however, infarct volumes showed no significant improvement, $33 \pm 8.7\%$ vs $24 \pm 9.9\%$, (control vs treated, $p > 0.05$) suggesting a neurorestorative effect, whereas in this study using aged rats, no significant ($p > 0.05$) improvement of functional outcome was observed, 15.2% in the ART, 18.2% in the mNSS and -9.3% in the FFT, yet a significant and substantial reduction in infarct volume was observed, $26.04 \pm 4.29\%$ vs $12.77 \pm 3.61\%$, (control vs treatment), $p < 0.05$, suggesting a neuroprotective effect, without the expected concomitant improvement in neurological outcome [13]. In neurorestorative therapy, the strategy is to treat the intact tissue to stimulate a repair process while in neuroprotection, the strategy is to treat the lesion itself and limit damage to the penumbral area. Neurorestorative agents act on intact parenchyma cells, including, neuroprogenitor cells to stimulate neurogenesis, angiogenesis, axonal sprouting and synaptogenesis [4]. Clinically, neurorestorative agents are more practical than neuroprotective agents, because the therapeutic drug targets intact parenchyma cells with intact cerebral blood flow removing time and delivery constraints. Neuroprotective agents, on the other hand, act on the damaged lesion and are administered

under 24 hours from time of stroke onset. Reduction of infarct volume does not always correlate with improvement of function. Many failed clinical trials were constructed with the premise that reduction of infarct volume in animals models would correlate to an improvement in functional outcomes in humans.

To investigate a possible explanation regarding the lack of functional improvement in these aged rats, we measured reactive gliosis to determine if any treatment effect could be observed. Astrocytic and microglial reactivity are increased in normal aged rats [2, 3]. After cerebral ischemia in aged rats, the increased reactivity of astrocytes and microglia contribute to an earlier development of the glial scar, which has been suggested to impede functional recovery [2]. Badan et al. showed that the glial scar peaks very early after transient ischemia in aged rats when compared to young rats and aged rats and were more severely impaired with a diminished functional recovery when compared with young rats even when infarct volumes showed no differences [25]. However, controversy exists whether reactive gliosis promotes or impedes recovery after stroke. Reactive gliosis after stroke encompasses a wide range of responses, producing inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) which impede neurite outgrowth and conversely, production of neurotrophic factors which promote regeneration [26]. Reactive astrocytes restrict the lesion and thus minimize the area of injury and inflammation, and studies in ischemic mice with knockout of GFAP, which exhibit diminished reactive gliosis, demonstrate a worse functional outcome than stroke in wild-type mice [27]. Although our data did not show a T β 4 treatment effect on reactive gliosis and thus a plausible explanation for the lack of recovery, we observed a significant correlation between reactive gliosis and improvement in neurological outcome involving both motor and sensory function (mNSS), thus supporting the concept that increased GFAP reactivity may be of benefit in improvement in functional outcome in the aged rat. Moreover, the lack of significant correlation between the FFT (cerebellar function), and GFAP immunoreactivity (as measured in the cerebral hemisphere) fits the anatomical relationship of improvement in function with the specific functional tests. Therefore, in our aged rat model, when only reactive gliosis is used as a variable, regardless of treatment or control group, the higher GFAP immunoreactivity, the better the outcome. Additionally, increased GFAP immunoreactivity was marginally inversely correlated with a reduction in infarction volume ($p=0.067$); also suggesting that reactive gliosis may limit the area of damage, as previously suggested [26, 27].

Evaluation of functional recovery in aging rats is complex [28]. Aged animals recover more slowly with reduced functionality when compared to younger animals [2]. The initial neurological deficits of the aged rats showed no significant differences in comparison to our previously published study using young rats, ART 113.2 ± 2.5 vs 115.1 ± 2.0 sec, FFT 32.0 ± 1.5 vs 30.5 ± 1.57 and mNSS 11.1 ± 0.25 vs 10.4 ± 0.40 , young vs aged, respectively. The functional improvement observed in young rats in our previously published study may possibly, in part, be attributed to the speed of the recovery [13]. The functional testing used in this study has only been validated in young rats and not aged rats. It is possible that the functional tests used in this study are not sufficiently sensitive to detect differences in neurological outcome. Motor function decreases significantly during aging and the movements required in these tests may require a more sensitive evaluation of motor function to detect differences between groups. A human analogy of this effect may be the ability of

elderly (>70 years) people to run a 100 yard dash quickly (most elderly could not run quickly or run at all) whereas younger adults (<20 years) could all run the race with speed, some faster, some slower enabling detection of differences between runners.

The reduction in infarct volume in the T β 4 treated group may be due to the marginally upregulated expression of cortical AQ4+EBA. AQ4 is predominately located in the foot process of the astrocytes and the increased expression along with EBA, a marker of endothelial cells, suggests a partial salvage of the BBB. Previous studies suggest that loss of AQ4 represents an active response of viable astrocytes and T β 4 may attenuate that loss [29]. The increase relative expression of cortical AQ4 supports the concept that the increased glial response after stroke may, at least play a role in the reduction of infarct volume in these aged rats.

The observation that T β 4 did not increase markers of myelination, OPCs, OL and MBP staining is consistent with previous research on aging progenitor cells. Here, we focused our study on white matter, as our preliminary work on T β 4 showed it to be a possible re-myelination agent [11, 30]. Age has a profound influence on disease recovery, as the ability of a mammalian tissue to replace mature cells declines with age [31]. A central hypothesis of aging is that homeostatic maintenance and regenerative potential of tissues decrease as an organism progresses through time [32]. Neurogenesis declines with increasing age in rats. Stroke induces neurogenesis, and in the aged rat the proliferative potential of the SVZ is conserved; however, the ability of the cells to migrate to the area of injury is diminished [33]. The majority of the decline (80%) occurs in the subgranular zone over 3 to 12 months of age [34, 35]. Many factors have been implicated in this decline. Most notably, is the reduced responsiveness of neural stem cells to environmental cues and the accumulation of inhibitory factors [36–38]. Certain diseases that commonly affect the elderly, such as osteoporosis or sarcopenia, are theorized to occur because cell loss exceeds renewal. Adult progenitor cells have become central to aging because of their ability to self-renew or engage in repair or replacement of diseased tissue. The aging process is associated with loss of self-renewal in adult stem cells and acquisition of defects in differentiation [31, 32]. These change over time of the plasticity and potentiality that are characteristic of adult progenitor cells are hypothesized to occur because of cumulative DNA mutations, oxidative damage, and accumulation and aggregation of abnormal proteins and lipids [31]. Therefore, the ability of a neurorestorative agent to stimulate recovery may be limited in the aged due to a lack of viable progenitor cells to induce a repair process.

Conclusions

Our data demonstrate that T β 4 administered to an aged rat model of embolic stroke 24 hours after MCAO results in no evident improvement in neurological functional outcome; however, a significant reduction of infarction was observed, suggesting a neuroprotective effect. These results in the aged rat are in contrast to our findings in younger animals in which neurological improvement was observed. Moreover, although we did not observe a treatment effect of T β 4 on reactive gliosis to explain the lack of functional improvement, we unexpectedly observed that reactive gliosis is highly correlated with functional outcome as

measured by the mNSS regardless of treatment group, supporting the premise that the reactive gliosis may be of benefit in recovery in aged animals.

Acknowledgments

Grant support: NIH, R01 AG038648. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. HFHS has a Material Transfer Agreement with RegeneRx Biopharmaceuticals Inc, Rockville, MD. A US Patent has been granted for use of T β 4 in neurological injury: PCT No. 9,149,509 Method for Improving Neurological Outcome after Neural Injury and Neurodegenerative Disease. Oct 6, 2015.

References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, 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, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB, C. American Heart Association Statistics and S. Stroke Statistics. Heart disease and stroke statistics—2015 update: a report from the American Heart Association. *Circulation*. 2015; 131(4):e29–322. <http://www.ncbi.nlm.nih.gov/pubmed/25520374>. [PubMed: 25520374]
2. Popa-Wagner A, Carmichael ST, Kokaia Z, Kessler C, Walker LC. The response of the aged brain to stroke: too much, too soon? *Curr Neurovasc Res*. 2007; 4(3):216–27. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17691975. [PubMed: 17691975]
3. Popa-Wagner A, Badan I, Walker L, Groppa S, Patrana N, Kessler C. Accelerated infarct development, cytogenesis and apoptosis following transient cerebral ischemia in aged rats. *Acta Neuropathol*. 2007; 113(3):277–93. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17131130. [PubMed: 17131130]
4. Zhang ZG, Chopp M. Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurol*. 2009; 8(5):491–500. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19375666. [PubMed: 19375666]
5. Chopp M, Li Y, Zhang ZG. Mechanisms underlying improved recovery of neurological function after stroke in the rodent after treatment with neurorestorative cell-based therapies. *Stroke*. 2009; 40(3 Suppl):S143–5. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19064763. [PubMed: 19064763]
6. Goldstein AL, Slater FD, White A. Preparation, assay, and partial purification of a thymic lymphocytopoietic factor (thymosin). *Proc Natl Acad Sci U S A*. 1966; 56(3):1010–7. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=5230175. [PubMed: 5230175]
7. Goldstein AL, Hannappel E, Sosne G, Kleinman HK. Thymosin beta4: a multi-functional regenerative peptide. Basic properties and clinical applications. *Expert Opin Biol Ther*. 2012; 12(1): 37–51. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22074294. [PubMed: 22074294]
8. Goldstein AL, Hannappel E, Kleinman HK. Thymosin beta4: actin-sequestering protein moonlights to repair injured tissues. *Trends Mol Med*. 2005; 11(9):421–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16099219. [PubMed: 16099219]
9. Zhang J, Zhang ZG, Morris D, Li Y, Roberts C, Elias SB, Chopp M. Neurological functional recovery after thymosin beta4 treatment in mice with experimental auto encephalomyelitis. *Neuroscience*. 2009; 164(4):1887–93. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19782721. [PubMed: 19782721]
10. Xiong, Y., Mahmood, A., Meng, Y., Zhang, Y., Zhang, ZG., Morris, DC., Chopp, M. Treatment of traumatic brain injury with thymosin beta(4) in rats. *J Neurosurg*. 2010. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20486893

11. Morris DC, Chopp M, Zhang L, Lu M, Zhang ZG. Thymosin beta4 improves functional neurological outcome in a rat model of embolic stroke. *Neuroscience*. 2010; 169(2):674–82. <http://www.ncbi.nlm.nih.gov/pubmed/20627173>. [PubMed: 20627173]
12. Wang L, Chopp M, Szalad A, Liu Z, Lu M, Zhang L, Zhang J, Zhang RL, Morris D, Zhang ZG. Thymosin beta4 promotes the recovery of peripheral neuropathy in type II diabetic mice. *Neurobiol Dis*. 2012; 48(3):546–55. <http://www.ncbi.nlm.nih.gov/pubmed/22922221>. [PubMed: 22922221]
13. Morris DC, Cui Y, Cheung WL, Lu M, Zhang L, Zhang ZG, Chopp M. A dose-response study of thymosin beta4 for the treatment of acute stroke. *J Neurol Sci*. 2014; 345(1–2):61–7. <http://www.ncbi.nlm.nih.gov/pubmed/25060418>. [PubMed: 25060418]
14. Zhang RL, Chopp M, Zhang ZG, Jiang Q, Ewing JR. A rat model of focal embolic cerebral ischemia. *Brain Res*. 1997; 766(1–2):83–92. <http://www.ncbi.nlm.nih.gov/pubmed/9359590>. [PubMed: 9359590]
15. Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniago P, Li Q, Lu M, Chopp M. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods*. 2002; 117(2):207–14. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12100987. [PubMed: 12100987]
16. Cenci MA, Whishaw IQ, Schallert T. Animal models of neurological deficits: how relevant is the rat? *Nat Rev Neurosci*. 2002; 3(7):574–9. <http://www.ncbi.nlm.nih.gov/pubmed/12094213>. [PubMed: 12094213]
17. Chen J, Zacharek A, Li Y, Li A, Wang L, Katakowski M, Roberts C, Lu M, Chopp M. N-cadherin mediates nitric oxide-induced neurogenesis in young and retired breeder neurospheres. *Neuroscience*. 2006; 140(2):377–88. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16580782. [PubMed: 16580782]
18. Lu M, Chen J, Lu D, Yi L, Mahmood A, Chopp M. Global test statistics for treatment effect of stroke and traumatic brain injury in rats with administration of bone marrow stromal cells. *J Neurosci Methods*. 2003; 128(1–2):183–90. <http://www.ncbi.nlm.nih.gov/pubmed/12948561>. [PubMed: 12948561]
19. S, II., editor. *User's Guide SAS/STAT 9.2*. 2010.
20. Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol*. 1998; 56(2):149–71. <http://www.ncbi.nlm.nih.gov/pubmed/9760699>. [PubMed: 9760699]
21. Lehrmann E, Christensen T, Zimmer J, Diemer NH, Finsen B. Microglial and macrophage reactions mark progressive changes and define the penumbra in the rat neocortex and striatum after transient middle cerebral artery occlusion. *J Comp Neurol*. 1997; 386(3):461–76. <http://www.ncbi.nlm.nih.gov/pubmed/9303429>. [PubMed: 9303429]
22. Gehrmann J, Bonnekoh P, Miyazawa T, Hossmann KA, Kreutzberg GW. Immunocytochemical study of an early microglial activation in ischemia. *J Cereb Blood Flow Metab*. 1992; 12(2):257–69. <http://www.ncbi.nlm.nih.gov/pubmed/1548298>. [PubMed: 1548298]
23. Sharma JC, Fletcher S, Vassallo M. Strokes in the elderly - higher acute and 3-month mortality - an explanation. *Cerebrovasc Dis*. 1999; 9(1):2–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9873157.
24. Fisher M, Albers GW, Donnan GA, Furlan AJ, Grotta JC, Kidwell CS, Sacco RL, Wechsler LR. Enhancing the development and approval of acute stroke therapies: Stroke Therapy Academic Industry roundtable. *Stroke*. 2005; 36(8):1808–13. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16020764. [PubMed: 16020764]
25. Badan I, Buchhold B, Hamm A, Gratz M, Walker LC, Platt D, Kessler C, Popa-Wagner A. Accelerated glial reactivity to stroke in aged rats correlates with reduced functional recovery. *J Cereb Blood Flow Metab*. 2003; 23(7):845–54. <http://www.ncbi.nlm.nih.gov/pubmed/12843788>. [PubMed: 12843788]
26. Liu, Z., Chopp, M. Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. *Prog Neurobiol*. 2015. <http://www.ncbi.nlm.nih.gov/pubmed/26455456>
27. Liu Z, Li Y, Cui Y, Roberts C, Lu M, Wilhelmsson U, Pekny M, Chopp M. Beneficial effects of gfap/vimentin reactive astrocytes for axonal remodeling and motor behavioral recovery in mice

- after stroke. *Glia*. 2014; 62(12):2022–33. <http://www.ncbi.nlm.nih.gov/pubmed/25043249>. [PubMed: 25043249]
28. Shapira S, Sapir M, Wengier A, Grauer E, Kadar T. Aging has a complex effect on a rat model of ischemic stroke. *Brain Res*. 2002; 925(2):148–58. <http://www.ncbi.nlm.nih.gov/pubmed/11792363>. [PubMed: 11792363]
 29. Friedman B, Schachtrup C, Tsai PS, Shih AY, Akassoglou K, Kleinfeld D, Lyden PD. Acute vascular disruption and aquaporin 4 loss after stroke. *Stroke*. 2009; 40(6):2182–90. <http://www.ncbi.nlm.nih.gov/pubmed/19372455>. [PubMed: 19372455]
 30. Santra M, Chopp M, Zhang ZG, Lu M, Santra S, Nalani A, Santra S, Morris DC. Thymosin beta 4 mediates oligodendrocyte differentiation by upregulating p38 MAPK. *Glia*. 2012; 60(12):1826–38. <http://www.ncbi.nlm.nih.gov/pubmed/23073962>. [PubMed: 23073962]
 31. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell*. 2008; 132(4):681–96. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18295583. [PubMed: 18295583]
 32. Rando TA. Stem cells, ageing and the quest for immortality. *Nature*. 2006; 441(7097):1080–6. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16810243. [PubMed: 16810243]
 33. Darsalia V, Heldmann U, Lindvall O, Kokaia Z. Stroke-induced neurogenesis in aged brain. *Stroke*. 2005; 36(8):1790–5. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16002766. [PubMed: 16002766]
 34. Bondolfi L, Ermini F, Long JM, Ingram DK, Jucker M. Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol Aging*. 2004; 25(3):333–40. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15123339. [PubMed: 15123339]
 35. Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci*. 1996; 16(6):2027–33. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8604047. [PubMed: 8604047]
 36. Li S, Carmichael ST. Growth-associated gene and protein expression in the region of axonal sprouting in the aged brain after stroke. *Neurobiol Dis*. 2006; 23(2):362–73. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16782355. [PubMed: 16782355]
 37. Li Y, Jiang N, Powers C, Chopp M. Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. *Stroke*. 1998; 29(9):1972–80. discussion 1980–1. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9731626. [PubMed: 9731626]
 38. Luo J, Daniels SB, Lenington JB, Notti RQ, Conover JC. The aging neurogenic subventricular zone. *Aging Cell*. 2006; 5(2):139–52. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16626393. [PubMed: 16626393]

Highlights

- Thymosin β 4 reduced infarct volume in aged embolic stroke rats.
- Thymosin β 4 did not improve functional outcome in aged embolic stroke rats.
- Reactive gliosis may be of benefit with regard to recovery in aged stroke rats.

Figure 1. Functional tests of control vs T β 4 treated rats. No significant differences were observed. On day 35, sticky tabs were reduced to half size on the adhesive test.

Figure 2.

Infarct volume of control vs T β 4 treated rats. A significant difference in lesion volumes was observed, 26.04 $\pm$ 4.29% and 12.77 $\pm$ 3.61%, (mean $\pm$ SE), $p < 0.05$

Figure 3.

Photograph shows NG-2+BrdU double-labeled cells in the IBZ. NG-2+BrdU double-labeled cells were not increased in the ipsilateral SVZ, IBZ and CC of Tβ4 (12mg/kg) treated rats when compared to control. White bar is 25 μ m in length. Arrow points to double-labeled cell.

Figure 4.

Photograph shows APC+BrdU double labeled cells in the IBZ. APC+BrdU double-labeled cells were not increased in the ipsilateral SVZ, IBZ and CC of Tβ4 (12mg/kg) treated rats when compared to saline control. White bar is 25 μm in length. Arrow points to double-labeled cell.

Figure 5.

Photograph shows MBP staining in the white matter bundles of the IBZ of control and Tβ4 (12mg/kg) treated rats. There is no increased MBP density in Tβ4 treated rats when compared to control. Black bar is 25 μm in length.

Figure 6.

Photograph shows GFAP staining in the IBZ of control and Tβ4 (12mg/kg) treated rats. Tβ4 treatment did not significantly alter GFAP staining when compared to control ($p=0.09$).

Black bar is 25 μ m in length.

Figure 7. Photograph shows AQ4+EBD double staining in the IBZ of control and Tβ4 (12mg/kg) treated rats. Tβ4 treatment marginally increased staining when compared to control ($p=0.056$). Arrows point to double-labeled area (AQ4+EBA). White bar is 25 μm in length.