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Enhancement of Neurogenesis and Memory by a Neurotrophic Peptide in Mild to Moderate Traumatic Brain Injury

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Abstract

Background—Traumatic Brain Injury (TBI) is a risk factor for Alzheimer disease (AD), a neurocognitive disorder with similar cellular abnormalities. We recently discovered a small molecule (Peptide 6) corresponding to an active region of human ciliary neurotrophic factor, with neurogenic and neurotrophic properties in mouse models of AD and Down syndrome.

Objective—To describe hippocampal abnormalities in a mouse model of mild to moderate TBI and their reversal by Peptide 6.

Methods—TBI was induced in adult C57Bl6 mice using controlled cortical impact (CCI) with 1.5 mm of cortical penetration. The animals were treated with 50 nmol/animal/day of Peptide 6 or saline for 30 days. Dentate gyrus (DG) neurogenesis, dendritic and synaptic density and AD biomarkers were quantitatively analyzed and behavioral tests were performed.

Results—Ipsilateral neuronal loss in CA1 and parietal cortex, and elevation of Alzheimer-type hyperphosphorylated tau and A-beta were seen in TBI-mice. When compared to saline, Peptide 6

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Dedicated to Dr. Inge Grundke-Iqbal who directed the original studies on Peptide 6, and who passed away unexpectedly on September 22, 2012.

SUPPLEMENTAL DIGITAL CONTENT

Supplemental Digital Content 1: Supplemental Methods.

Supplemental Digital Content 2: Figure: Elevation of Alzheimer-type biomarkers in TBI mice. Immunohistochemical staining of hippocampus and parietal cortex (A) revealed elevation of hyperphosphorylated tau and Aβ, two key hallmarks of Alzheimer disease in ipsilateral versus contralateral regions (B).

Red: PHF1 staining against phosphor-tau at serine 396/404 (Scale bar = 100 μm, inset scale bar = 10 μm); Green: 4G8 staining against APP and Aβ (Scale bar = 50 μm)

*(p<0.05), **(p<0.01), ***(p<0.001), one way ANOVA, Bonferroni's *post hoc* test

treatment increased number of newborn neurons, but not uncommitted progenitors, in DG by 80%. Peptide 6 treatment also reversed TBI-induced dendritic and synaptic density loss while increasing activity in tri-synaptic hippocampal circuitry, ultimately leading to improvement in memory recall on behavioral testing.

Conclusion—Long-term treatment with Peptide 6 enhances the pool of newborn neurons in DG, prevents neuronal loss in CA1 and parietal cortex, preserves dendritic and synaptic architecture in the hippocampus, and improves performance on a hippocampus-dependent memory task in TBI mice. These findings necessitate further inquiry into therapeutic potential of small molecules based on neurotrophic factors.

Keywords

Ciliary Neurotrophic Factor; Dentate Gyrus; Memory; Neurogenesis; Neural Stem Cells; Therapeutics; Traumatic brain injury

INTRODUCTION

Traumatic Brain Injury (TBI) is the leading cause of death and disability in children and adults from ages 1 to 44[1] with an annual incidence of 1.7 million in the US, causing enormous economic burden[1–3] and no proven therapy to improve long-term outcomes[4, 5]. Owing to the inherent vulnerability of hippocampus to trauma, cognitive impairment is widely accepted as the most devastating deficit in long-term survivors of TBI. In fact, hippocampal neuronal loss accompanies >80% of fatal TBI,[6] and apoptotic events in the hippocampus can be observed up to 12 months following TBI[7]. Observations from experimental models of TBI indicate enhanced progenitor cell proliferation in the hippocampal dentate gyrus (DG) following TBI and is widely considered an endogenous repair mechanism to counter cognitive decline after brain trauma[8, 9]. The biological drive behind increased progenitor cell proliferation in the hippocampus is based, in part, on neurotrophic factor dynamics within the local microenvironment[10, 11]. Consequently, increasing adult hippocampal neurogenesis and stimulating neuronal plasticity pharmacologically is considered a very useful strategy towards inhibiting cognitive decline following TBI[12–15]. While administration of neurotrophic factors has generated much excitement in the literature[16, 17], adverse effects and difficult pharmacokinetics have limited the clinical usefulness of this approach[18]. We previously reported the development of an 11-mer peptide based on a biologically active region of human Ciliary Neurotrophic Factor (CNTF)[19]. This peptide, Peptide 6, was shown to have significant neurogenic and neurotrophic effects in the DG of normal adult C57BL6 mice[19], as well as transgenic mouse models of Down syndrome and Alzheimer disease (AD), disorders with documented abnormal hippocampal neurogenesis and cognitive impairment[20, 21]. Given the parallels between chronic hippocampal degeneration of Alzheimer-type and acute hippocampal disruption in trauma, particularly neurotrophic factors imbalance[22–26], we hypothesized a similar neurogenic effect of Peptide 6 in a mouse model of TBI. Here, we report that chronic administration of Peptide 6 to a controlled cortical impact (CCI)- mouse model of mild-to-moderate TBI resulted in increased neuronal differentiation of progenitors in the DG. Furthermore, 30-day administration of Peptide 6 ameliorated TBI-induced decrease in

dendritic and synaptic density in DG and CA1 regions of the hippocampus. There was also significant increase in the immunoreactivity of immediate-early gene zif268 in the CA1 region, indicating increased neuronal activity and activation of the traditional excitatory trisynaptic pathway in the hippocampus. These findings were translated into measurable improvement in memory on behavioral testing. This study necessitates further inquiry into therapeutic potential of novel small molecules based on neurotrophic factors.

METHODS

Pertinent methods are described below. Detailed materials and methods including TBI induction, treatment and behavioral paradigms, animal sacrifice, tissue processing, antibodies, stereology and statistical methodology are detailed in the Supplemental Methods (see Supplemental Digital Content 1). These studies were conducted under protocol # 100954 of the Institutional Animal Care and Use Committee of the University of New Mexico. Scientists who were blinded to the treatment performed all stereological counts and behavior testing.

Induction of TBI in mice

Induction of TBI in mice was based on the CCI model as described previously[27]. Briefly, with the head fixed in a stereotaxic frame, a midline skin incision was performed and a 5 mm left lateral craniotomy was made using a motorized drill. CCI was induced with an impact device using a 3 mm diameter metal tip with a velocity of 5 m/sec to a penetration depth of 1.5 mm below the dura. The impact was centered at 2.7 mm lateral to midline and 3.0 mm anterior to lambda. The craniotomy site was sealed with Loctite Gel glue (Henkel Corp., Rocky Hill, CT) and the scalp was sutured. The mice were given antibiotic prophylaxis with 500 units/gm of IM Bicillin (Pfizer, NY) peri-operatively and 0.01 mg/kg twice daily of buprenorphine (Reckitt Benckiser Pharmaceuticals, N Chesterfield, VA) for three days for comfort.

Administration of BrdU and Peptide 6

To study neurogenesis, animals were given intraperitoneal (i.p.) injections of Peptide 6 (50 nmol/animal/day) or saline (control) for 30 days. Mice in either treatment group received twice-daily injections of BrdU (75 mg/kg, Sigma-Aldrich, St. Louis, MO) for two days beginning one-day post trauma (Figure 1A–B).

Stereology & confocal microscopy

Neurogenesis was assessed in the DG by counting the number of BrdU-immunoreactive (BrdU-IR) and BrdU-NeuN-IR cells in various layers of the DG. The granular cell layer (GCL) was subdivided into an inner and outer half (iGCL and oGCL). The iGCL consisted of the subgranular zone (defined as a 2–3 nuclei thick layer bordering the GCL) and the inner half of the GCL adjacent to the Hilus (Hil); the outer GCL (oGCL) was defined as the half of the GCL adjacent to the molecular layer (Mol) as described previously[28]. To assess cell proliferation, Brdu-IR cells were counted in every 5^{th} section using a 40× oil objective of a Nikon 90i fluorescent microscope equipped with a Nikon C1 3-laser confocal system. Employing principles of unbiased stereology, the optical fractionator method was used to

estimate cell counts for the DG[29]. For each brain, at least 100 cells were counted based on coefficient of error determinations. To assess neuronal maturation, BrdU-NeuN-IR cells were counted using 100× oil objective in every 10th section. To ensure objectivity, z-stacks were collected for each double IR cell and analyzed later by generating maximum projection and 3-D constructs. A cell was counted only when it showed double IR on 3-D reconstructed images.

MRI acquisition

MRI was performed on a 4.7 Tesla dedicated research MR scanner (Bruker Biospin, Ettlingen, Germany), equipped with a small-bore linear radiofrequency coil (72 mm). All data processing was performed using in-house developed software written in 64-bit MATLAB (Mathworks, Natick, MA, USA) running on a UNIX machine. Initial localizer images were acquired followed by T2-weighted (T2WI) and diffusion-weighted images.

Behavioral Testing

Behavioral testing included spatial reference memory test in the Morris Water Maze (MWM)[30] as described previously[19] and detailed in the Supplemental Methods (see Supplemental Digital Content 1).

Statistics

Data are presented as mean with standard error of mean (SEM). Kolmogorov-Smirnov test was used to determine normality of the data. For stereological studies an unpaired Student's t-test was used. For immunohistochemical analysis involving multiple groups, ANOVA with post hoc Bonferroni's test was used. Further intergroup comparisons involving the effect of TBI and/or treatment were performed using unpaired Student's t-test. For behavioral studies, either one-way or repeated measures ANOVA tests were used with post hoc Tukey or Bonferroni tests where appropriate. Statistical outliers were detected using Grubbs test for normally distributed data. Differences with $p < 0.05$ were considered significant.

RESULTS

Anatomical extent of brain injury in TBI-mice

Imaging studies performed 6 weeks after induction of TBI revealed extent of structural damage to the brain. Although the depth of brain deformation was kept at 1.5mm, T2W MRI showed involvement beyond the contusion, including hippocampus and its subregions: DG, CA1 and CA3 regions (Figure 1C–D). Nissl staining revealed a smaller and shrunken ipsilateral hippocampus compared to contralateral side (Figures 1E and 5A). There was obvious shrinkage of ipsilateral parietal somatosensory cortex on Nissl staining (Figure 1E).

Proliferation of Neural Progenitors in TBI-mice treated with Peptide 6

There was no significant change in the number of BrdU immunoreactive (BrdU-IR) cells, i.e. new born progenitors, in the granule cell layer (GCL) of DG on chronic administration of Peptide 6 following trauma (Figure 2). Although stereological analysis of the number of progenitors in sub-regions of the DG revealed an increase in the number of BrdU-IR cells in

the subgranular zone (SGZ) in Peptide 6 treated animals when compared to the saline control group (Figure 2B and Table 1), this did not reach statistical significance (mean ± SEM: 3303 ± 765 control, 4850 ± 568 Peptide 6, p=0.11, Student's t-test).

Similarly, stereological counts did not reveal any significant increase of BrdU-IR cells in the inner GCL (iGCL) of TBI-mice treated with Peptide 6 when compared to saline (3197 ± 380 versus 2100± 595, p=0.11, Student's t-test) or outer GCL (oGCL, 1203 ± 220 versus 1588 ± 224, p=0.25, Student's t-test; Figure 2 B and E, Table 1). Together, these data suggest that Peptide 6 did not increase neural progenitor cell proliferation in the GCL of DG.

Neuronal differentiation of progenitor cells in the dentate gyrus

More than half of newly born progenitors die before maturation (a process that takes at least three weeks[31]); net neurogenesis in the DG is, therefore, determined by the number of progenitors that survive as mature neurons[32]. In order to determine whether Peptide 6 induced differentiation of DG progenitors into mature neurons, we counted the number of BrdU-IR cells expressing the mature neuronal marker NeuN in the GCL of the DG, i.e. Brdu-NeuN-IR cells after 30 days of injury (Figure 2 and Table 2). We found an 80% increase in BrdU-NeuN-IR cells in Peptide 6 treated TBI-mice when compared with the saline control group (mean ± SEM: 1901 ± 265 versus 1057 ± 217, p= 0.036, Student's t-test; Figure 2 C–D and Table 2). Sub region analysis of GCL again revealed focal and specific increase in treatment induced newborn neurons in the iGCL (1225 ±169 versus 648± 176, p=0.03, Student's t-test) but not in oGCL (658± 124 versus 409 ± 64, p=0.13, Students' t-test; Figure 2D and F, Table 2).

The number of TUNEL positive cells in DG on the side ipsilateral to injury was significantly decreased by Peptide 6 treatment (~18% decrease, mean ± SEM: 18 ± 0.8 versus 15 ± 1, p=0.048, Student's t-test; Figure 2G and H).

Together, this suggests that Peptide 6 caused a surge in the number of newborn mature neurons without increasing the number of progenitors. Whether this was due to better survival of new neurons or an increase in neuronal fate commitment of progenitors (or both) is unclear from these data, although the overall number of apoptotic events in the DG was significantly decreased with peptide treatment.

Aberrant migration or "ectopic birth" of progenitors in the dentate gyrus

A relatively small number of newborn cells (20–25%) are found in the oGCL (or migrating towards the molecular layer), while the rest remain in iGCL[33]. A significant increase in number of progenitors in the oGCL is considered abnormal and has been implicated in abnormal connectivity[34] such as that seen in Schizophrenia[35]. We therefore, counted the number of BrdU-IR in the iGCL and oGCL separately and calculated an "ectopic birth index" (EBI=oGCL/GCL) as described previously (Figure 2 E–F)[19, 34].

EBI analysis revealed that 40% of newborn cells in the saline treated group were found in the oGCL compared with 32% in Peptide 6 treated animals (Figure 2E and Table 1). Although this did not reach statistical significance (p=0.08, Student's t-test), it does suggest that an abnormally high proportion of new born cells are found in the oGCL in response to

TBI, and that perhaps there is evidence, albeit weak, to suggest that Peptide 6 treatment corrects this aberrant migration or “ectopic birth”. A similar, yet non-significant, trend was also seen in newborn mature neurons (43% in saline treated versus 34% in Peptide 6 treated, p=0.15, Student’s t-test; Figure 2F and Table 2).

TBI-induced reduction in hippocampal dendritic and synaptic density and its recovery by Peptide 6

We further investigated the effect of TBI on dendritic network and synapses in injured mice by measuring expression of MAP2 (a dendritic marker) and synaptophysin (a synaptic marker). Quantification of fluorescence intensity in DG (including molecular layer, granule cell layer, and hilus), CA3 and CA1 sub-regions revealed site and side-specific decreases in post -TBI mice (Figure 3). MAP2 expression was significantly decreased in all three hippocampal regions ipsilateral to the side of injury compared to the contralateral side (Bonferroni’s post-hoc test, $p<0.001$ for DG, CA3, and CA1; Figure 3B). Peptide 6 treatment increased MAP2 staining in the ipsilateral DG, CA3, and CA1 regions (an increase of 28.2%, 8.1%, and 21.1% respectively compared to ipsilateral saline treated TBI hippocampus). However, this difference was only statistically significant in the DG region (Bonferroni’s post-hoc test, $p>0.05$, Student’s *t*-test, $p=0.04$; Figure 3B, left panel) and a trend was evident in the CA1 region (Bonferroni’s post-hoc test, $p>0.05$, Student’s *t*-test, $p=0.06$). Moreover, in Peptide 6 treated TBI mice, MAP2 intensity in DG did not differ significantly between ipsilateral and contralateral sides (Figure 3B left panel). In the CA1 region, MAP2 expression was significantly higher on the contralateral side in Peptide 6 treated mice than the saline treated group (Figure 3B, right panel, Bonferroni’s post-hoc test, $p<0.001$).

TBI-induced reduction in hippocampal synaptic density and its recovery by Peptide 6

Synaptophysin expression was also significantly decreased in ipsilateral DG (Bonferroni’s post-hoc test, $p>0.05$, Student’s *t*-test, $p=0.02$) but not in CA3 and CA1 regions compared to the contralateral side in saline treated TBI mice (Figure 4 A–B). Similar to MAP2 data, synaptophysin expression was also increased by 27.2%, 23.5%, and 40% in DG, CA3, and CA1 regions, respectively, on the ipsilateral side in Peptide 6 treated mice compared to controls; the differences were either statistically significant or a strong trend was evident (Figure 4B; DG, Bonferroni’s post-hoc test, $p>0.05$, Student’s *t*-test, $p=0.037$; CA3, Bonferroni’s post-hoc test, $p>0.05$, Student’s *t*-test, $p=0.09$; CA1, Bonferroni’s post-hoc test, $p<0.05$) Together, these findings suggest a “rescue effect” of Peptide 6 on TBI-induced decrease in the level of MAP2 and synaptophysin in various regions of the hippocampus.

Neuronal loss in ipsilateral hippocampus and parietal cortex

We next sought to determine neuronal loss in hippocampus and parietal cortex at 6 weeks post injury (Figure 5). Quantitative cell counts of Nissl stained neurons revealed an 18% neuronal loss in the ipsilateral CA1 region of hippocampus in saline treated animals compared to the contralateral side (83 ± 5.7 versus 98 ± 7.8, Bonferroni’s post-hoc test, $p<0.05$, Figure 5A). Interestingly, in Peptide 6 treated TBI-animals, this neuronal loss was significantly reduced in the ipsilateral CA1 region when compared with saline treated animals (100.5 ± 8.3 versus 83 ± 5.7, Bonferroni’s post-hoc test, $p<0.05$, Figure 5A–B).

There was a 37% loss of neurons in the ipsilateral parietal cortex, site of primary impact of TBI, compared to the contralateral side in saline treated animals (94 ±1.2 versus 129 ± 5.7, Bonferroni's post-hoc test, $p<0.05$, Figure 5C–D). Similar to CA1 region, in Peptide 6 treated TBI-animals, neuronal loss was significantly stunted in the ipsilateral cortex when compared to saline treated mice (94 ±1.2 versus 126 ± 12, Bonferroni's post-hoc test, $p>0.05$, Student's t-test, p=0.03, Figure 5D).

Immediate-early gene expression in hippocampal circuitry

Changes in expression of immediate early genes (IEGs) such as FBJ osteosarcoma gene (c-fos), activity regulated cytoskeletal –associated protein (Arc) and early growth response 1 (Egr1 or Zif268) have been shown to be strongly coupled to neuronal activity associated with learning and memory,[31, 36] and their expression in new born cells is detectable by 3–4 weeks[37, 38]. We, therefore, measured zif268-IR in hippocampus of control and treated TBI-mice that were sacrificed within 3 hours of behavioral testing (Figure 5E–F). Although DG and CA3 regions did not reveal any differences, we found an increase of over 129% in zif268-IR in the CA1 region of TBI-mice treated with Peptide 6 when compared with controls, indicating increased activity in the traditional tri-synaptic pathway of hippocampal circuitry (Fig. 5F; Student's *t*-test, $p<0.001$).

Elevation of Alzheimer-related biomarkers in TBI-mice

Mild-to-moderate TBI is a risk factor for later development of AD[39–43]. In order to assess if TBI-mice displayed molecular abnormalities of AD, we measured the relative immunofluorescence of hyperphosphorylated tau and Aβ, two key hallmarks of AD. Quantitative immunofluorescence of PHF1 staining (against tau phosphorylated at serine 396 and 404) revealed significant elevation of hyperphosphorylated tau in ipsilateral CA1 region in both saline and Peptide 6 treated TBI mice (15% and 30% increase respectively, Bonferroni's post-hoc test, $p<0.05$, [see Figure, Supplemental Digital Content 2]). Similarly, staining with 4G8 (against Aβ and APP) revealed significant elevations in ipsilateral parietal cortex and CA1 regions (14% and 22% increase, respectively, Bonferroni's post-hoc test, $p<0.05$) compared to contralateral regions in saline treated TBI mice (see Figure, Supplemental Digital Content 2). There was no significant effect of Peptide 6 treatment on these AD-related biomarkers (Bonferroni's post-hoc test, $p>0.05$).

Behavioral impairment in TBI-mice and the effect of Peptide 6 in cognitive recovery

We evaluated the effect of Peptide 6 on cognition using a spatial reference memory test in the Morris water-maze (MWM). This spatial reference memory task assesses hippocampus-dependent reference memory in rodents, requiring that mice use a spatial navigational strategy to find a fixed submerged escape platform. For this purpose, we tested mice before injury and treatment (saline or Peptide 6) to establish baseline performances. The same mice were then injured and treated (saline or Peptide 6) and underwent another set of testing on the MWM (Figure 6A). This allowed us to study the effect of Peptide 6 treatment on TBI mice. Although MRI T2 signal abnormalities extended medially into the secondary, and possibly, primary motor area, there was no evidence of gross motor abnormality on swimming speeds between the injured and sham groups (Figure 6B).

All groups learnt equally well under both testing conditions. Pre and post TBI testing revealed no differences in escape latencies during training phase of the MWM, i.e. in both instances, escape latencies decreased from day 1 to day 4 of training ($p>0.05$, repeated measures ANOVA, Figure 6C).

During probe trial, there was significant improvement in latency to target, percent time and percent distance travelled in the target quadrant in Peptide 6 treated animals when compared to saline treated TBI mice ($p<0.01$, one-way ANOVA, post hoc Tukey, Figure 6D–F).

DISCUSSION

Cellular response to injury in the hippocampus

Moderate TBI induces rapid necrotic death of immature neurons in the hippocampus[44] while mature granule neurons are largely spared[45–47]. There is an enhanced level of cellular proliferation in key neurogenic areas of the brain following a variety of insults including stroke and trauma[8, 48–50]. A greater than 10-fold increase in BrdU-IR cells in the DG was reported in a lateral fluid percussion model of TBI[8]. The rate of production of new cells in the DG is reported to peak between 3 to 7 days after injury and returns to basal level in 2 weeks[9], with new cells predominantly being microglia/macrophages and astrocytes at 72 hours of injury[8]. There is evidence suggesting that even if new neurons are formed, they never survive to an age where they would be of functional consequence in the normal hippocampal circuitry[31].

A more recent study suggested no net increase in neurogenesis in a CCI mouse model of moderate TBI despite an increase in number of progenitors in the DG[51] amounting to a “failed innate response”. This state of impaired neurogenesis is coupled with a robust inflammatory response that persists in a chronic fashion[52]. It is likely that these progenitors proliferate in an altered microenvironment which impairs their differentiation potential and/or integration in the local circuitry[8, 12, 48]. In fact, levels of various neurotrophic factors correlate strongly with outcome in pediatric severe TBI[53].

Parallels between TBI and AD

Mild to moderate TBI causes neurodegeneration of the type seen in AD[54–57] and is considered a risk factor for AD in humans[40–43]. In fact, there are many parallels between AD and TBI hippocampus at the cellular and molecular levels. For example, in both TBI and AD hippocampus, there is alteration in the levels of FGF2, NGF and BDNF[10, 11, 23, 24, 58–62]. Moreover, like TBI[63], there is profound loss of dendritic and synaptic density in AD[20]. The occurrence of neurofibrillary degeneration, however, is extremely difficult to demonstrate in rodents, which over the natural course of their lives, never develop classical AD pathology[64, 65]. This Alzheimer-type neurofibrillary degeneration is only seen in transgenic mice overexpressing mutated forms of human tau and or APP and at much older ages[65–70]. The findings of elevated Aβ and hyperphosphorylated tau in ipsilateral CA1 regions of TBI mice in the current study are, therefore, both interesting and remarkable.

Effect of TBI on hippocampal dendritic and synaptic density

It is reported that within 3 hours of CCI induced injury in rodents, there is profound loss of MAP2 immunofluorescence in the apical dendrites of pyramidal neurons in the ipsilateral cortex that extends beyond the area of contusion[63, 71]. It is interesting to note that despite neuronal cell death in the hippocampus following TBI, the degree of cognitive impairment does not match with the amount of cell death. Even though mature granule neurons are largely spared[45–47], there is dramatic drop in the density of dendritic spines and synapses in the surviving mature hippocampal neurons in moderate TBI after 72 hours of injury[47]. Our data confirms significant decreases in MAP2 staining throughout the hippocampus, including DG, CA1 and CA3 regions and similar decreases in synaptophysin levels in DG at 30 days following injury. This suggests that these changes persist in a chronic fashion and are likely to be involved in long-term cognitive deficits.

Behavioral performance of TBI mice

We evaluated the effect of Peptide 6 on cognition using a spatial reference memory test in the MWM, the most frequently utilized protocol to study hippocampus dependent spatial learning and memory in rodents[30]. The hippocampus processes information about the relationships among distal environmental cues into a spatial map where spatial coordinates of the submerged platform are encoded[30]. The hippocampus is also crucial for memory storage, consolidation and restitution of the spatial information[72]. This test was especially appropriate because TBI did not cause impairment in swim speeds in these mice. Pre injury training on the MWM showed no group differences in learning (Figure 6C), establishing baseline uniformity in spatial encoding. This was maintained in the post TBI training phase with no differences between saline and Peptide 6 treated mice. However, on probe trial, we found a significant improvement in measures of memory retention in Peptide 6 treated animals when compared with saline treatment.

CA1 susceptibility in TBI

Of the various hippocampal sub regions, CA1 is most vulnerable to hypoxic and ischemic insults[73, 74] when compared to DG and CA3[75–77]. In this study, we saw elevations of Alzheimer-type biomarkers only in the CA1 region. Although we found significant decrease in synaptic and dendritic densities in all regions of the hippocampus, neuron cell loss was only significantly seen in the CA1 region. This was not only prevented with Peptide 6 treatment, its effect on MAP2 and synaptophysin IR was most profound in the CA1 region. Consequently, there was also an upregulation of immediate-early gene expression in the CA1 region of the hippocampus suggesting that mature CA1 neurons were actively participating in hippocampal dependent spatial paradigms. In MWM, activation of CA1 and CA3 regions is temporally and functionally distinct during different phases of the test[7879]. For example, when CA3 is experimentally blocked, direct activation of CA1 place cells might be sufficient for retrieval of spatial information during probe test[79], suggesting a predominant role of CA3 in learning phase and of CA1 in recall. It is, therefore, not surprising that in the current study, TBI-mice did not display learning deficits but were impaired in recall.

Neurotrophic factor replacement in TBI and challenges in clinical utility

Supplementing the hippocampus with neurotrophic factors that drive stem cells towards differentiation and sustain local microenvironment is a powerful concept in neuropharma[53]. Several studies have reported enhancement of neurogenesis in TBI models through neurotrophic factor supplementation such as S100B, erthyropoietin, EGF and FGF or combination therapy like human umbilical cord blood cells and granulocyte colony stimulating factor with resultant superior performance in memory tasks[12–15, 80]. While the administration of neurotrophic factors has generated much excitement in the literature, invasive mode of delivery, adverse side effects and difficult pharmacokinetics have very much limited the clinical usefulness of this approach[18]. Consequently, there are efforts now to develop small compounds mimicking neurotrophic factors that can cross the blood brain barrier and can be given peripherally[81, 82].

Peptide 6 and its potential clinical applications

The ciliary neurotrophic factor (CNTF) is a major determining factor for neurogenesis, both in the hippocampus and subventricular zone[33, 83]. Like other neurotrophins[18], the therapeutic potential of exogenous CNTF is eclipsed by its short half-life when administered peripherally, requiring invasive mode of administration with unpredictable pharmacokinetics[84]. CNTF is a large protein (22.7 kDa) which when given peripherally, only weakly reaches the CNS, has unpredictable pharmacokinetics and a very short plasma half life,[84] and hence, must be given directly into the brain. The novelty and the significance of Peptide 6 lies in the use as a potentially “druggable” compound, resembling a biologically active region of CNTF, which can be administered peripherally and is effective at nanomolar levels. Our previous study has shown that Peptide 6 is blood–brain barrier permeable, has a plasma half-life of over 6 h and enhances neurogenesis and promotes neuronal plasticity in the hippocampus[19]. Our long-term studies with Peptide 6 (up to 3 months in 3×Tg-AD mice[20]) or its fragment, P21 (up to 12 months[85]) did not demonstrate any side effects (such as anorexia, hyperalgesia and weight loss) associated with the parent molecule CNTF.

Peptide 6 contains a putative leukemia inhibitory factor receptor (LIFR) binding sequence of CNTF, the D1 cap region[86], and acts as an antagonist of the tripartite CNTF receptor complex[19]. This action of Peptide 6 inhibits leukemia inhibitory factor (LIF) activity and JAK/STAT3 signaling mediated by this receptor[19]. LIF is known to inhibit neurogenesis and promote self-renewal of the early, mostly gliagenic, progenitor cells[87–89]. Therefore, we speculate that the inhibition of LIF by Peptide 6 enhances neurogenesis by promoting the differentiation of the neural precursor cells through CNTF signaling[33, 89].

Now in a mild-to-moderate TBI mouse model, we show that Peptide 6 enhances differentiation of newly born progenitors in the dentate gyrus 30 days after injury. We believe that this neurogenic effect of Peptide 6 is due to its partial inhibition of LIF activity by directly binding to the D1 cap region of LIF receptor[19]. The fact that there was no statistical increase in the number of progenitors suggests a specific effect of Peptide 6 in promoting neuronal maturation and survival that is not seen naturally after TBI[9, 51]. Importantly, the newborn mature neurons remain within the physiological confines of the GCL and display no ectopic migration or birth, a phenomenon linked with epilepsy and

schizophrenia[34, 35]. We saw a robust correction of dendritic and synaptic markers in key regions of the injured hippocampus suggesting that Peptide 6 provides a neurotrophic milieu that might help sustain local microenvironment after mild-to-moderate brain injury.

CONCLUSION

Here, we demonstrate enhancement of neurogenesis by a small molecule in a mouse model of mild to moderate TBI. We further demonstrate that chronic administration of this molecule resulted in recovery of dendritic density and synaptic loss and activation of the traditional tri-synaptic memory pathway in the hippocampus of injured mice. This also resulted in improvement in memory on behavioral testing. The fact that this molecule is neurogenic and is given peripherally and non-invasively demonstrates its potential in hippocampal regeneration in the post-traumatic brain. Our data further emphasizes the role of neurotrophic factor supplementation in TBI and makes a strong case for neurotrophic factor-based novel therapies for the injured hippocampus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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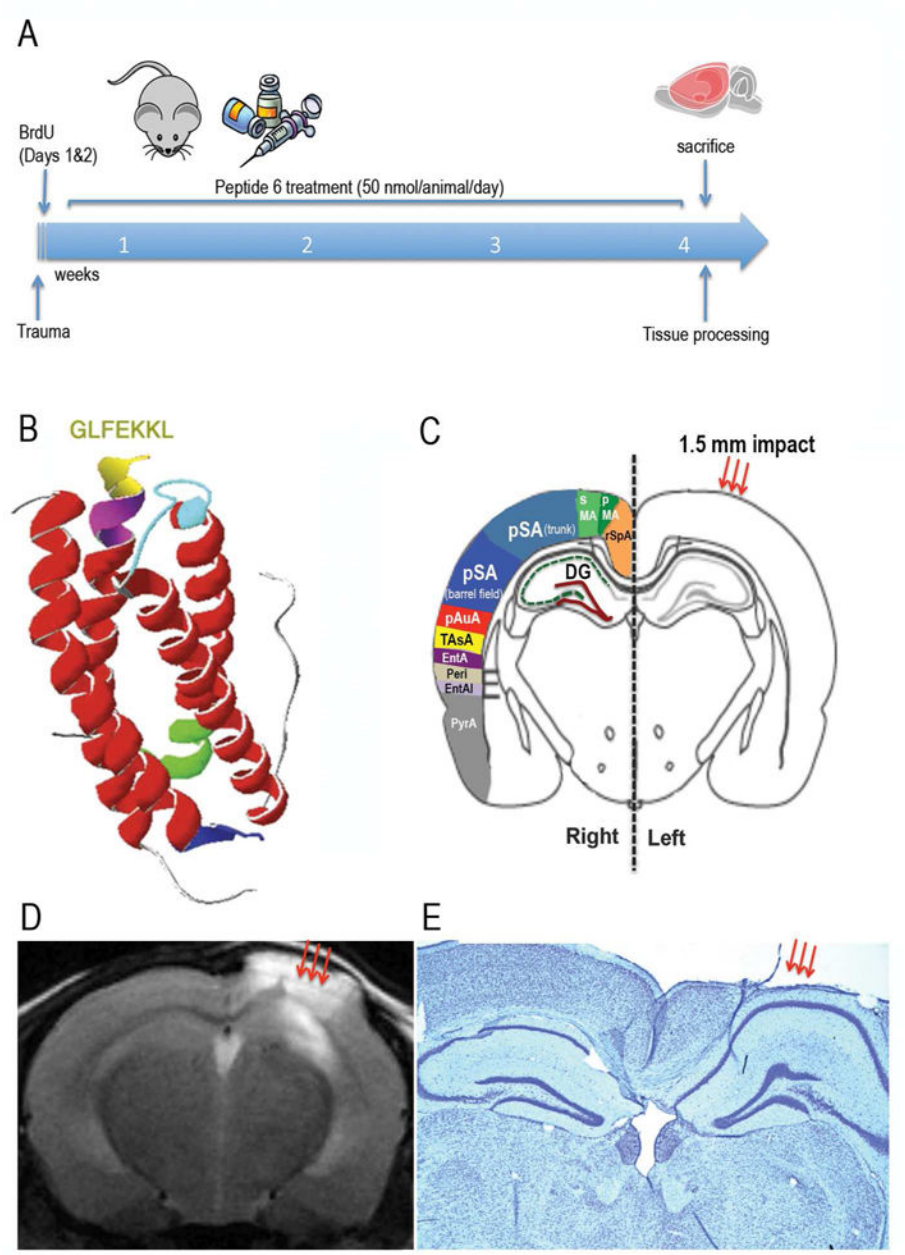

Figure 1. Experimental paradigm and extent of injury

(A) Experimental paradigm. C57BL6 female mice were subjected to controlled cortical impact (CCI) of 1.5 mm deformation and received twice daily injections of BrdU (75 mg/kg) for two days and either saline or Peptide 6 (50 nmol/animal/day), one day after injury for 30 days. Mice were sacrificed on day 30. (B) CNTF is an α-helical molecule with secondary structure consisting of four anti-parallel α -helices. Five regions (represented in different colors) correspond to the epitopes of neutralizing anti-human CNTF antibodies (adapted with permission from Chohan et al. 2011[19]). Peptide 6 corresponds to VGDGGLFEKKL epitope and contains the critical D1 cap region-binding site of CNTF receptor complex.

(C) Schematic representation of mouse brain, coronal section, with side of injury (arrows, left) and colored areas depicting functional regions (right). (D) A representative T2W MRI sequence of a TBI mouse 30 days after CCI injury. Note that although brain deformation was kept at 1.5 mm, T2 signal change was seen beyond the contusion and into the hippocampal area. (E) Nissl staining depicting typical cytoarchitectural changes in the TBI-mouse 30 days after injury (10× magnification).

rSpA: retrosplenial area; pMA: primary motor area; sMA: secondary motor area; pSA: primary sensory area; pAuA: primary auditory area; TAsA: temporal association area; EntA: entorhinal area; Peri: perirhinal area; EntAl: lateral entorhinal area; PyrA: pryiform area; DG: dentate gyrus.

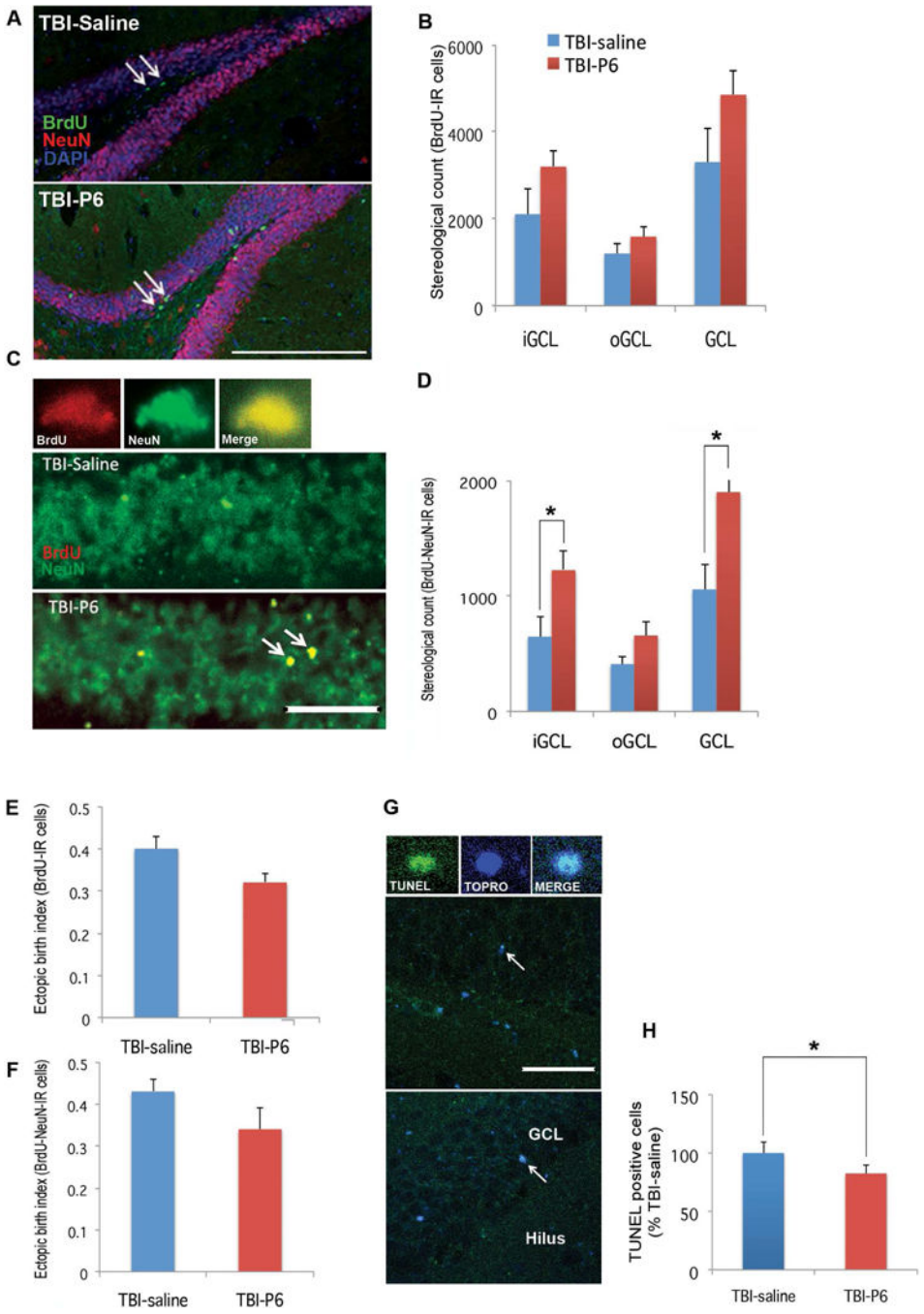

Figure 2. Neural progenitor cell proliferation and neurogenesis in the TBI mouse

(A) Progenitor cell proliferation in the DG of a TBI mouse 30 days after injury (green: BrdU, red: NeuN, blue: DAPI). (B) There was no statistical difference between the number of new born progenitors (BrdU-IR cells) in saline and Peptide-6 treated mice. (C) New born neurons in the granule cell layer of the DG of a TBI-Peptide 6 treated mouse. The top three panels show identity of a newborn neuron (red: BrdU, green: NeuN, yellow: merge). (D) Chronic treatment with Peptide 6 significantly increased the number of newborn neurons (BrdU-NeuN-IR cells) in the DG of TBI mice. (E & F) “Ectopic birth index” analysis

suggested that up to 40% of newborn cells and 43% of newborn neurons were located beyond the iGCL of DG in TBI-saline mice suggesting “ectopic birth” and/or aberrant migration. Although not statistically significant, this number was lower in Peptide 6 treated group (32% and 34% respectively).

(G & H) TUNEL staining in DG to assess apoptotic events. Top three panels in (G) represent identity of a cell undergoing apoptosis (green: TUNEL, blue: TOPRO). There was an ~18% decrease in the number of TUNEL positive cells in the DG ipsilateral to injury in Peptide 6 treated mice when compared to saline treated animals.

All scale bars =100**μ**m.

BrdU (5′bromodeoxyuridine), BrdU-IR (BrdU immunoreactive), oGCL (outer granule cell layer), iGCL (inner granule cell layer), DG (dentate gyrus). Statistical analysis done using Student’s t-test with p value <0.05 (*).

“Ectopic birth index” calculations were performed using the formula (oGCL/GCL) according to Donovan et al., 2006[34].

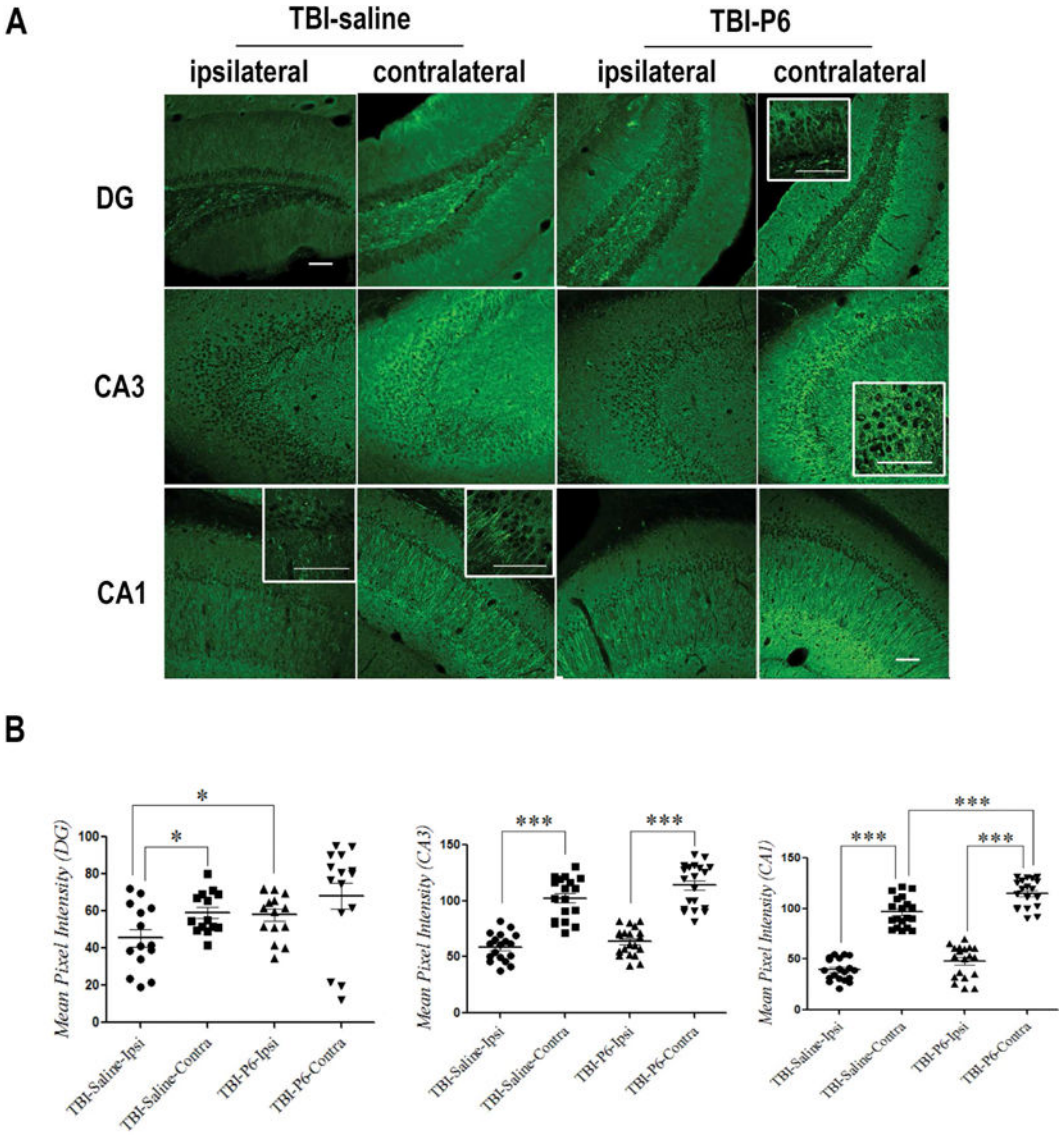

Figure 3. Loss of dendritic density in TBI hippocampus and its recovery by Peptide 6

(A) Photomicrographs illustrating MAP2 immunoreactivity in different areas of the hippocampus in saline and Peptide 6 treated TBI-mice. (B) There was a significant loss of dendritic density in the ipsilateral DG, CA3 and CA1 subregions of TBI-mice hippocampus compared to contralateral regions. (B, left panel) Chronic treatment with Peptide 6 increased MAP2 staining by 28% in DG ipsilateral to the injury as compared to saline treated mice. A similar, but non-significant, trend was also observed in CA3 (B, middle panel) and CA1 (C, right panel) regions in Peptide 6 treated mice. Scale bar = 100 μm. Insets show high magnification images (acquired with 100× objective)

MAP2 (microtubule associated protein 2), TBI-P6 (TBI-Peptide 6 treated group)

* ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), one way ANOVA, Bonferroni's post-hoc test or Student's t-test.

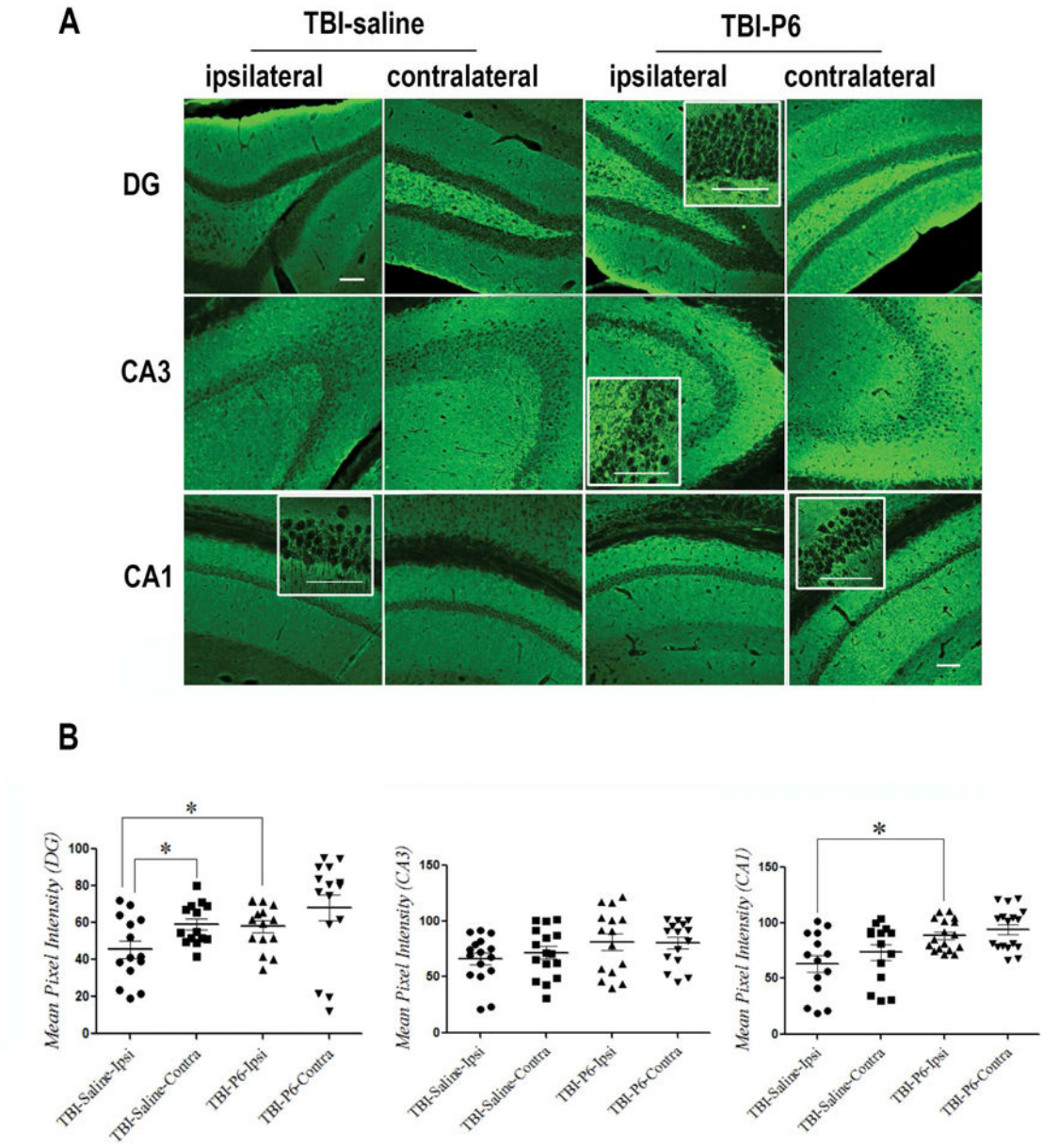

Figure 4. Synaptic loss in TBI hippocampus and its recovery by Peptide 6

(A) Photomicrographs illustrating synaptophysin immunoreactivity in different areas of the hippocampus in saline and Peptide 6 treated mice. (B) Compared to contralateral side, there was significant decrease in synaptophysin immunoreactivity in ipsilateral DG but not CA3 or CA1 regions of TBI-mice hippocampus. There was a 27%, 23% and 40% increase in synaptic density in DG (B, left panel), CA3 (B, middle panel) and CA1 (B, right panel) regions on the ipsilateral side in Peptide 6 treated TBI-mice as compared to saline treated animals. Scale bar = 100 μm. Insets show high magnification images (acquired with 100× objective).

DG (dentate gyrus), TBI-P6 (TBI-Peptide 6 treated group)

* ($p<0.05$), one way ANOVA, Bonferroni's post-hoc test, or Student's t-test.

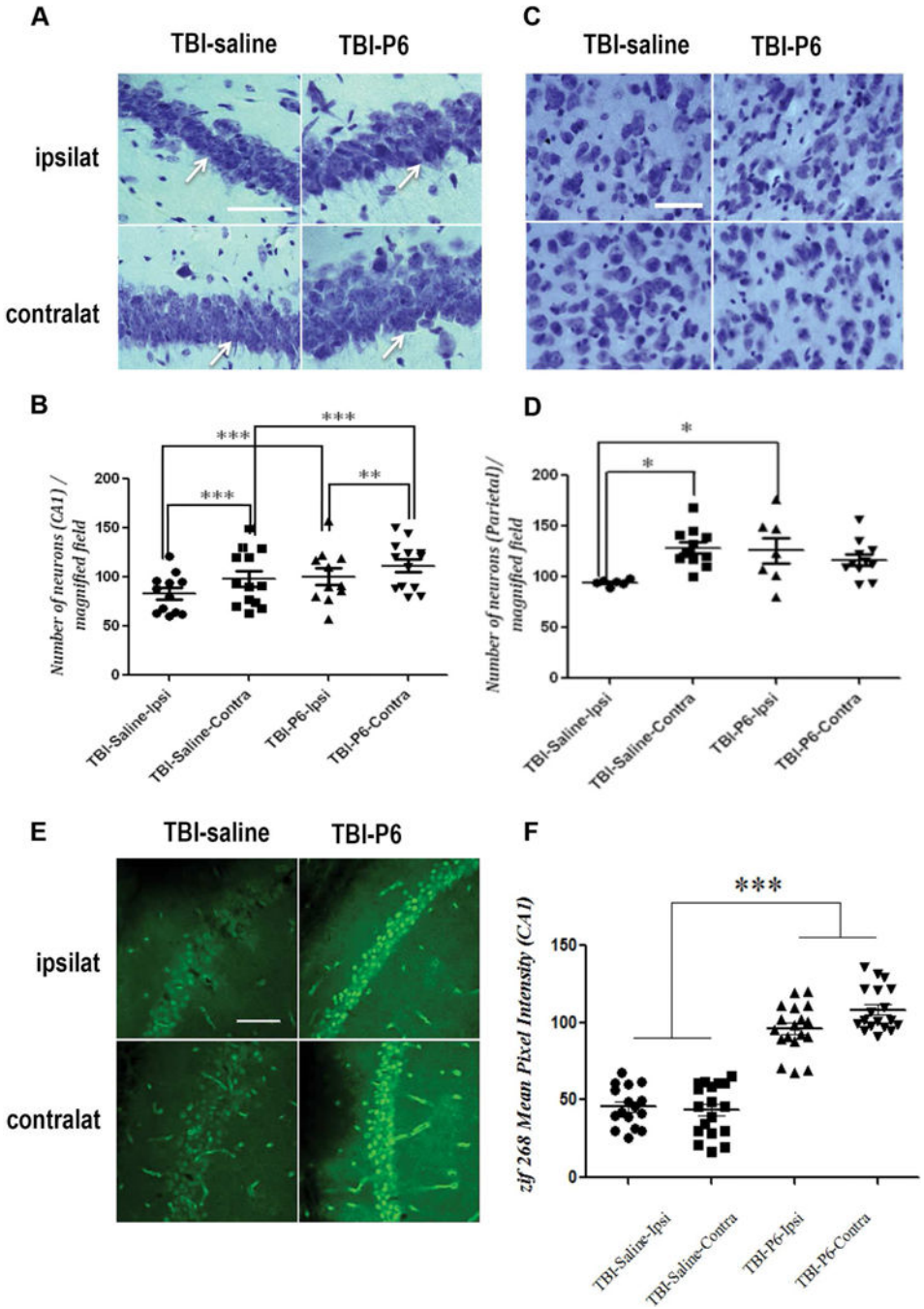

Figure 5. Cortical neuronal loss and Immediate-early gene expression in hippocampus

(A–D) Photomicrographs demonstrating Nissl staining in the CA1 region (A) and parietal cortex (C). There was significant loss of neurons in the ipsilateral CA1 region (B) and parietal cortex (D) in saline treated TBI mice compared to contralateral side. Chronic treatment with Peptide 6 prevented this loss. Arrows in (A) show visibly shrunken DG in saline treated animals. (E) Photomicrographs illustrating immediate-early gene expression (zif 268) in the CA1 region of saline and Peptide 6 treated TBI-mice, 30 days after injury and within 3 hours of performance in a hippocampus-dependent memory task. There was an

almost 130% increase in the levels of zif268 in CA1 region of Peptide 6 treated TBI-mice as compared to saline treated animals in both ipsilateral and contralateral sides (F). Scale bar = 50 μm in (A and C) and 100 μm in (E)

zif268 (early growth response 1 gene), TBI-P6 (TBI-Peptide 6 treated group)

***($p<0.001$), Student's t-test

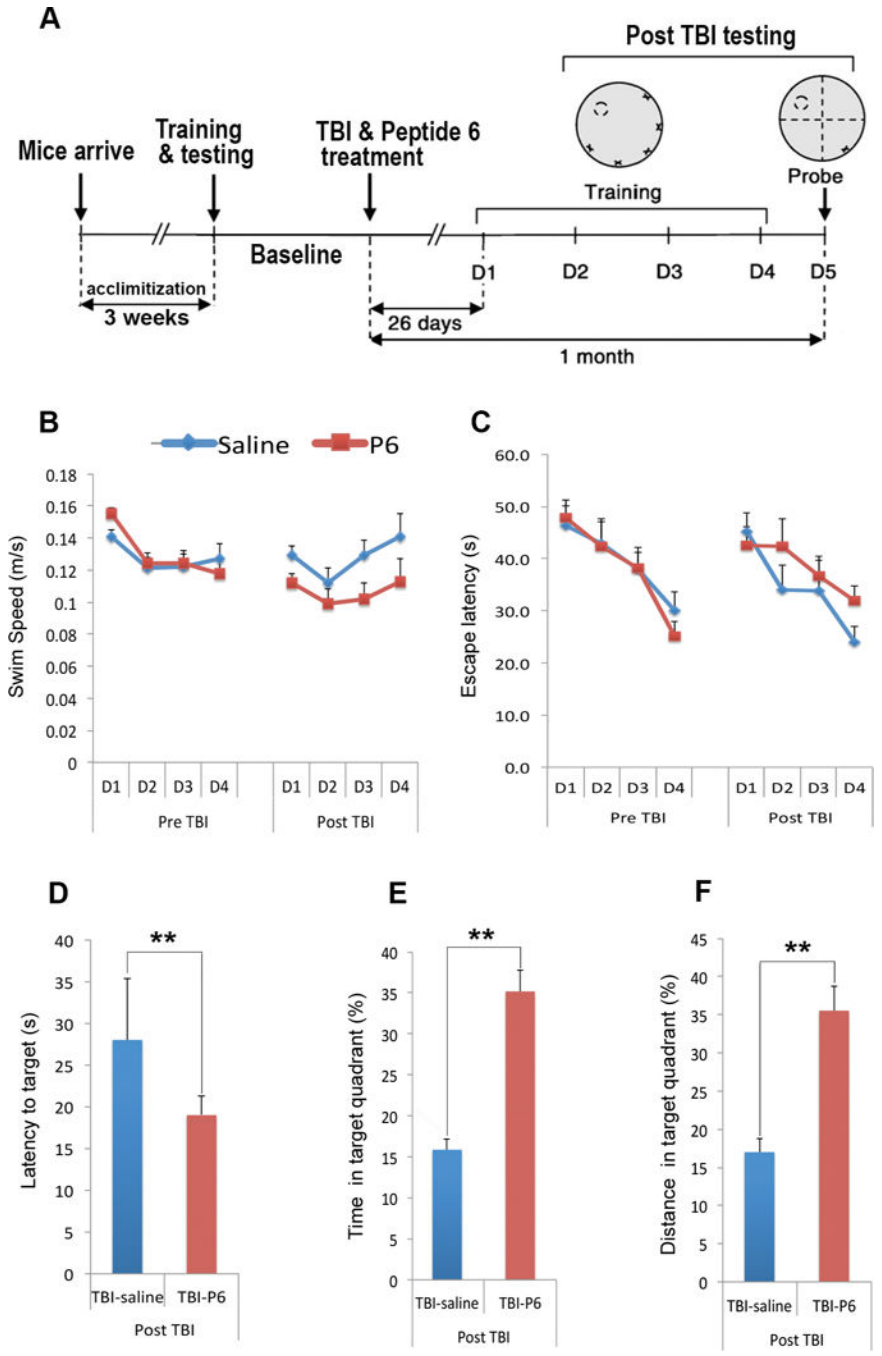

Figure 6. Behavioral testing on Morris Water Maze

(A) Behavioral testing paradigm on Morris Water Maze (MWM). After 3 weeks of acclimatization, all animals underwent 4 day training on the MWM to establish baseline learning. The mice were retrained on day 26 after TBI and treatment (saline or Peptide 6) with the probe trial on day 30. (B) There was no difference in pre or post TBI swim speeds between the various groups. (C) All animals showed equivalent learning on MWM as evidenced by decreasing escape latencies from day 1 to day 4 (repeated measures ANOVA). Additionally, there was no effect of Peptide 6 on learning in TBI animals. (D) On probe trial,

Peptide 6 treatment caused significant improvement in latency to target, (E) percent time spent in target quadrant and (F) percent distance covered in target quadrant.
TBI-P6 (TBI-Peptide 6 treated group), D1 (day 1)
** ($p<0.01$) one-way ANOVA, post hoc Tukey

Table 1

Stereological counts (mean ± SEM) of newborn progenitors (BrdU-IR cells) in various layers of the dentate gyrus

	TBI-saline	TBI-Peptide 6	p value
GCL	3303(765)	4850(568)	0.11
iGCL	2100(595)	3197(380)	0.11
oGCL	1203(220)	1588(224)	0.25
EBI (0GCL/GCL)	0.40(0.03)	0.32(0.02)	0.08

GCL: granule cell layer, iGCL: inner granule cell layer, oGCL: outer granule cell layer, EBI: ectopic birth index

p-value based on unpaired Student's t-test

Table 2

Stereological counts (mean ± SEM) of newborn neurons (BrdU-NeuN-IR cells) in various layers of the dentate gyrus

	TBI-saline	TBI-Peptide 6	p value
GCL	1057(217)	1901(265)	***0.03****
iGCL	648(176)	1225(169)	***0.03****
oGCL	409(64)	658(124)	0.13
EBI(0GCL/GCL)	0.43(0.03)	0.34(0.05)	0.15

GCL: granule cell layer, iGCL: inner granule cell layer, oGCL: outer granule cell layer, EBI: ectopic birth index

p-value based on unpaired Student's t-test (*p<0.05)