Molecular Neurobiology (2019) 56:8420–8434
https://doi.org/10.1007/s12035-019-01680-z

Neurotrophic Factors Mediated Activation of Astrocytes Ameliorate Memory Loss by Amyloid Clearance after Transplantation of Lineage Negative Stem Cells

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Received: 2 March 2019 / Accepted: 14 June 2019 / Published online: 27 June 2019

Abstract

Alzheimer's disease (AD) is one of the untreatable neurodegenerative disorders with associated societal burden. Current therapies only provide symptomatic relief without altering the rate of disease progression as reported by Lanctot et al. (Therapeutic Advances in Neurological Disorders 2 (3):163–180, 2009). The increased number of failed clinical trials in last two decades indicates the imperative need to explore alternative therapies for AD as reported by Tuszynski et al. (Nature Medicine 11 (5):551–555, 2005) and Liyanage et al. (Alzheimer's & Dementia 4:628–635, 2005). In this study, we aimed to decipher the role of neurotrophic factors in the reversal of memory loss by transplantation of lineage negative (Lin-ve) stem cells in a male mouse model of cognitive impairment induced by intrahippocampal injection of amyloid β-42 (Aβ-42). The efficacy of human umbilical cord blood (hUCB) derived Lin-ve stem cells were analyzed by neurobehavioral parameters, i.e., Morris water maze and passive avoidance after bilateral intra-hippocampal transplantation using stereotaxic surgery. Real-time PCR and immunohistochemistry was carried out in brain tissues in order to analyze the expression of neurotrophic factors, apoptotic, astrocytic, and other neuronal cell markers. The transplantation of Lin-ve stem cells led to reversal of memory loss associated with reduction of Aβ-42 deposition from the brains. The molecular analysis revealed increase in neurotrophic factors, i.e., glial derived neurotrophic factor (GDNF), ciliary derived neurotrophic factor (CNTF), and Brain-derived neurotrophic factor (BDNF) after transplantation. The administration of ANA-12, a TrkB inhibitor, reversed the behavioral and molecular effects of stem cell transplantation suggesting involvement of BDNF-TrkB pathway in the rescue of memory loss. We believe that the amyloid clearance results from activation of astrocytes and anti-apoptotic pathways added by neurotrophic factors.

Keywords Alzheimer's disease · Neurotrophic factor · BDNF · Umbilical cord blood · Lineage negative stem cells · Amyloid injury · Memory loss

Significance Statement

Umbilical cord blood (UCB) banking has increased manifold across the world without corresponding data generation for its preclinical testing and its efficacy. Since Alzheimer's disease (AD) is an untreatable neurological disorder and various drug trials have failed successively, this study unravels the preclinical efficacy of UCB-derived stem cells and underlying molecular mechanisms involved in rescue of amyloid pathology as well as memory loss. The data presented in this paper provides compelling evidence to show that UCB-derived lineage negative stem cells can upregulate BDNF which is the chief cause of rescue of memory loss caused due to artificially delivered Amyloid β in mouse model of brain injury. Hence, our study paves way for clinical trials for utilization of cord blood for the treatment of AD.

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Introduction

Alzheimer's disease (AD) is an untreatable neurodegenerative disorder which affects almost 29.8 million individuals worldwide [4]. It accounts for almost 60–70% cases of Dementia. Currently FDA-approved drugs, acetylcholinesterase inhibitors (donepezil) and NMDA receptor antagonists (memantine), provide symptomatic relief without relieving disease pathology [5–7]. In last decade, drugs that were found to be promising in pre-clinical studies by clearing amyloid load from AD mouse models, failed during clinical trials [8, 9]. Several factors which were argued to influence the negative outcomes in clinical trials, ranged from unavailability of suitable animal models to genetic and phylogenetic variabilities between human and rodents [10]. Hence, these failures call for reinvigorated efforts to test new biotherapeutic approaches in AD. Cellular therapies in pre-clinical studies have repeatedly shown potential disease modifying effect in AD models. For example, transplantation of various sources of stem cells has shown cognitive improvement in murine models of AD [11, 12]. Several reports including our previous studies have shown varying effects of stem cell transplantation. These therapeutic benefits could either include replacement or integration of degenerating neurons by stem cells aided by paracrine effects through release of neurotrophic factors, immunomodulation and migration [13–16]. However, there is a lack of clear understanding of underlying molecular mechanisms in the rescue of function effects of these cellular therapies.

In most of the recent findings, it is believed that transplanted stem cells exert non-redundant neuroprotective effects mediated by neurotrophic factors such as BDNF, GDNF, and CNTF [14]. Brain-derived neurotrophic factor (BDNF) is an essential neurotrophic factor which acts by BDNF-TrkB pathway for synaptic regulation [17]. In AD patients, lower levels of BDNF have been reported in postmortem brains [18] suggesting its critical role in the disease. Similarly, GDNF and BDNF levels have been found to be significantly reduced in serum of AD patients [19]. In transgenic (3xTg-AD) aged AD mice, transplantation of neural stem cells has been shown to result in improvement of cognitive dysfunction by increased hippocampal synaptic density mediated by BDNF [20].

The association of GDNF with AD is rarely studied; however, depletion of these factors appear to be linked to AD pathologies [21]. GDNF levels have been reported to be significantly low in serum of AD patients whereas in few studies, it has been concomitantly increased in the cerebrospinal fluid [22]. A dysregulated pattern of tissue expression of GDNF was found in postmortem middle temporal gyrus (MTG) of AD [23]. In 3xTg-AD mice, the downregulation of GDNF has been reported and this effect was ameliorated by 6-month voluntary exercise [24]. Furthermore, the overexpression of GDNF, using lentiviral vector for 6 months in 3xTg-AD mice, has been shown to improve learning and cognitive memory consolidation by upregulation of BDNF without alleviating amyloid pathology [25]. Interestingly, the interaction between BDNF induced TrkB signaling and GDNF-induced RET pathway has also been shown by inhibition of either TrkB receptor or RET using RNAi, which confirms their cross-talk [26].

Ciliary neurotrophic factor (CNTF) has also been shown to exert neuroprotective effect in the management of neurodegenerative disorders [27]. It is expressed in astrocytes of subventricular zone/dentate gyrus region in interface with neurogenic niche. Its receptors (CNTFRα) are present on neuronal progenitors as well as in hippocampal neurons. CNTF plays a fundamental role in hippocampal neurogenesis besides exerting its neurogenic effects in subventricular region [28, 29]. CNTF is known to regulate adult neurogenesis, enhance proliferation, and neuroblast formation mediated by dopaminergic activity [29]. The peripheral administration of peptide-6, which is an active region of CNTF in 3xTg-AD has shown enhanced neurogenesis in dentate gyrus and increased hippocampal neuronal plasticity which is possibly linked to cognitive improvement [30]. Expectedly, the localized delivery of recombinant cells secreting CNTF improved cognitive performance by stabilizing the synaptic proteins [31]. The various in vivo and in vitro studies suggest the dominant role of CNTF in astrocyte activation [32–36]. In this respect, it is pertinent to note that the insoluble Aβ plaque formation is one of the major symptoms of AD and astrocytes play a major role in its clearance from brain parenchyma into perivascular space [37]. Therefore, it is imperative that therapy targeting such putative neurotrophic factors may provide superior cognitive outcomes by alleviating AD pathology.

Our recent study showed that CD117 and CD34 stemness of hUCB Lin-ve stem cells reverse memory loss in mice intrahippocampally injected with aggregated Aβ-42 peptides [13]. We further aimed to investigate downstream molecular mechanisms underlying the therapeutic effect of hUCB Lin-ve stem cells transplanted in this mouse model. Our results reveal that the neurobehavioral improvement exerted by the Lin-ve stem cells was mediated by neurotrophic factors associated activation of anti-apoptotic pathway at the site of injury. This was evidenced by alteration of neurotrophic factors such as BDNF, GDNF, and CNTF in experimental mice after stem cell transplantation. Hence, we speculated that transplanted stem cells could trigger the release of endogenous neurotrophic factors and consequently alleviate Aβ-induced damage and perhaps delay apoptosis in AD brain causing neurobehavioral improvement.

Material and Methods

Study Design

The experimental procedures are supplementary to our previously published article [14]. Six to eight-week old Swiss albino male mice were used for experiment after approval from Institute Animal Ethical Committee (75/IAEC/473). Mice were anesthetized using intra-peritoneal injection of Xylazine Hydrochloride (50 mg/ml) and Ketamine Hydrochloride (50 mg/ml) in the ratio of 1:4. The memory loss was established by intra-hippocampal delivery of oligomeric form of Aβ-42. The Lin-ve SC were transplanted after 21 days of Aβ-42 induced injury. The memory was assessed by neurobehavioral analysis (Morris water maze and passive avoidance) 10-day post-transplantation. ANA-12 (1 mg/kg body weight) (Sigma) is an effective TrkB receptor inhibitor which causes cognitive impairment [38, 39]. It was intraperitoneally administered 1 h before the start of behavioral analysis daily from day 31 to day 41 in Aβ-42 injury + Lin-ve SC transplanted mice. Mice were sacrificed using high dose of xylazine and ketamine on day 41 after completion of all the behavioral analysis. The experimental timeline is shown in Fig. 1 (reused and modified from Parul Bali et al. 2018 with permission) [13].

Mouse Model of Amyloid Pathology and Memory Loss

Amyloid β (1-42) aggregates were prepared from 0.1 mg stock of Aβ-42 protein fragment (Sigma-Aldrich, USA) and incubated at 37 °C for 4 days and 4 °C for 6 h. Stereotaxic surgery was performed to inject 5 μL of amyloid solution into hippocampal region of mice brains. Mice were fixed over stereotaxis apparatus to restrict any head movement. Bilateral craniotomy was done using 26G needle after exposing the skull. Bregma zero was located and micro-syringe was placed at a specific axis for hippocampal delivery, i.e., anteroposterior (AP) + 2 mm, mediolateral (ML) ± 2 mm and dorsoventral (DV) − 2.5 mm. One micromolar of Aβ-42 was injected at a controlled speed of 1–2 μL/min and the needle was retained for 5–7 min for proper diffusion. The skin was sutured back and neosporin was applied for antiseptic. Mice were kept into a single cage and observed until they recovered. Similarly, 1xPBS was injected bilaterally for vehicle control.

Isolation and Purification of hUCB Lin-ve Stem Cells

hUCB Collection

hUCB collection was done in accordance with ethical guidelines as approved by Institutional Committee on Stem Cell Research (IC-SCR) (Approval no. PGI-IC-SCR-67-2015/1654). The blood was collected from umbilical cord with placental tissue of newborn deliveries (aged between 20 and 35 years) at gestation period of ≥ 28 weeks after obtaining patient's consent. The donors were screened and following conditions were excluded, i.e., hepatitis B infection, HIV infection, syphilis infection, untreated urinary tract infection, acute infection, unclean vaginal examination, fever, prolonged rupture of membrane (> 24 h), foul smelling amniotic fluid, and major congenital malformation in the new born.

The cord was clamped using mosquito forceps to prevent further blood loss. The vein of cord was located and blood was withdrawn using 21G needle with 50-ml syringe. Blood was immediately transferred into EDTA (anticoagulant)-

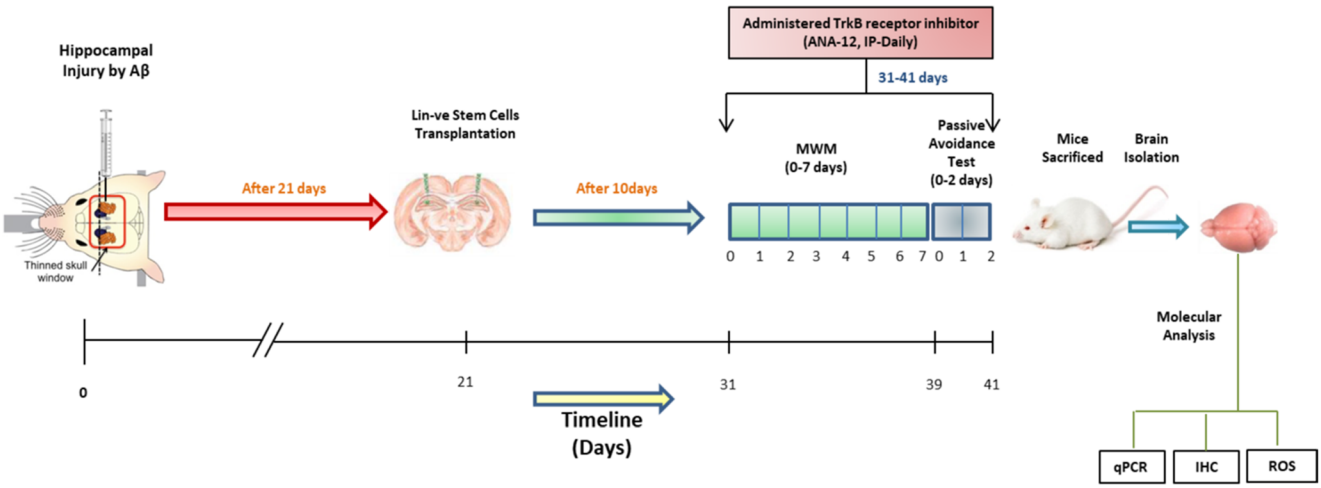

Fig. 1 Schematic representation of in vivo study design and timeline. The timeline of experiments starting at 0 day point with amyloid injury by stereotaxic surgery ending at the 41st day by sacrificing the mice for end-point analysis has been represented. After 21 days of amyloid injury, Lin-ve stem cells were transplanted in a treatment group and neurobehavioral analysis was carried out at 10-day post-transplantation. In another group, ANA-12 (TrkB Inhibitor) was administered during neurobehavioral analysis. The end-point analysis was done by real-time PCR, immunohistochemistry and ROS analysis

containing vials and transferred in ice for further processing for Lin-ve stem cell isolation.

Lin-ve Stem Cell Isolation

The hUCB was layered over the Ficoll Histopaque (Sigma-Aldrich, USA) in 1:1 ratio and subjected to density gradient centrifugation in a swing bucket centrifuge (REMI Lab. Instruments, India) at 1500 rpm for 30 mins for lymphocyte separation. The buffy coat containing lymphocytes appeared at ficoll plasma interface after centrifugation and was subsequently collected. It was mixed and washed with equal volume of MACS-BSA (1:50) buffer. The mononucleated cells (MNCs) were subjected to magnetic activated cell sorting (MACS) for Lin-ve cell enrichment using human Lin-ve isolation kit (Miltenyi Biotech, Germany). Lineage depletion kit contained biotinylated monoclonal antibodies for markers viz. CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A). MNCs were incubated with the antibodies cocktail and then to streptavidin coated magnetic microbeads. The cells were then subjected to magnetic separation by passing it through column under strong magnetic field. Lineage positive cells, i.e., committed to specific cell type remained in the column because of magnetic beads, whereas unbound Lin-ve SC passed through it. The enriched Lin-ve stem cells were then kept at 4 °C until transplanted into mouse model.

Labeling and Transplantation of Lin-ve SC

Lin-ve stem cells were labeled using CFDA-SE (Vybrant CFDA cell tracer kit, Invitrogen, USA) dye, which diffuses passively into the cells. It is a colorless dye which gives green fluorescence after the action of cellular esterase. Cells were incubated in 5–10 μM of CFDA-SE solution at 37 °C for 15 min. Cells were then washed with PBS to exclude excess dye from the cell suspension. CFDA-SE labeled 50,000 cells were transplanted in the intra-hippocampal region bilaterally using stereotaxic surgery. The recruited cells (CFDA tagged) were tracked under fluorescence microscope using FITC filter.

Behavioral Analysis

Morris Water Maze

The long-term spatial memory loss was assessed by Morris water maze (MWM) assay. MWM consisted of a circular water tank, artificially divided into four quadrants, with the target platform kept hidden in the quadrant. The protocol was executed for 7 days. It consisted of 6 acquisition days and one retrieval day (7th day). The mice were subjected to each of four quadrants and the time taken to locate the hidden platform was recorded. The mice behavior was assessed and recorded by video camera kept over the tank, which was connected to ANY-maze software. The escape latency of mice was estimated for six acquisition days and compared to amyloid injury group, stem cell-transplanted group and TrkB inhibitor injected group. On retrieval day (day7), the time spent by mice in each quadrant and the mean distance from the platform (i.e., search error) was measured.

The experimental design for MWM varies depending on the question we ask. In some studies, the animals are exposed to MWM after amyloid injection followed by therapeutic treatment in the same cohort [40]. Others train the mice first before injury. We used the first paradigm in order to examine the effect of stem cell transplantation on acquisition and retrieval in the Amyloid injured mice. We have used this well-standardized protocol in our previous studies.

Passive Avoidance Test

Passive avoidance is a fear motivated test used for short term memory assessment. Mice neurobehavior was contrary to their innate tendencies for disposition to dark chamber. In conditioning phase, mice were kept in light chamber and received mild foot shock of 20 mA for 2–3 s, as soon it entered the dark chamber. In the test phase, i.e., after 24 h, the latency to cross the door was calculated.

Molecular Analysis

Real-time PCR

cDNA was prepared from RNA extracted from test and control brains using RNeasy Lipid Tissue Mini kit (Qiagen). Real-time PCR was performed to estimate the expression of genes using primers (Sigma) for BDNF, CNTF, GDNF, GFAP, Bcl2, Caspase3, SV2A, Ki67, and VEGF in 96-well plate using StepOnePlus system (Applied Biosystems, USA). The expression and fold changes were analyzed using (Step one, Applied Biosystems) software and each sample was normalized to its β-actin expression. The details of primer sequences are listed in Table 1.

The list of markers analyzed in the study and their primer sequences used in real-time PCR.

Immunohistochemistry

The immunohistochemistry (IHC) of hippocampal region was carried out in the 6–7-μm-thick brain sections obtained from Cryostat (Leica CM 1510S) at − 24 °C. The sections for IHC were fixed using Histochoice (Sigma) and the blocking was done using 5% BSA (Sigma). Sections were incubated with anti-mouse primary antibodies in 1:100 dilutions at 4 °C, overnight. Sections were then incubated with rabbit or goat raised secondary antibodies in 1:200 dilution at room

Table 1 List of forward and reverse primers used for qPCR analysis

Sr. no.	Gene	Primer sequence	
1	GDNF	Forward	5′-TGGGCTATGAAACCAAGG-3′
		Reverse	5′-CAACATGCCTGGCCTACT-3′
2	GFAP	Forward	5′-ACAGACTTTCTCCAACCTCCAG-3′
		Reverse	5′-CCTTCTGACACGGATTTGGT-3′
3	SV2A	Forward	5′-GTCTTTGTGGTGGGCTTTGT-3′
		Reverse	5′-CGAAGACGCTGTTGACTGAG-3′
4	VEGF	Forward	5′-CTACTGCCGTCCGATTGAGAC-3′
		Reverse	5′-GGCTTGTCACATCTGCAAGTAC -3′
5	BDNF	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
6	CNTF	Forward	5′-GCGAGCGAGTCGAGTGGTTGTCTG-3′
		Reverse	5′-TTAGCTTTCGGCCACCAGAGTGGAGA ATTC-3′
7	Ki67	Forward	5′-CAGTACTCGGAATGCAGCAA −3′
		Reverse	5′- CAGTCTTCAGGGGCTCTGTC-3′
8	Bcl-2	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
9	Caspase3	Forward	5′-ATTCAGGCCTGCCGGGGTAC-3′
		Reverse	5′-AGTTCTTTCGTGAGCATGGA-3′

temperature for 30 mins. Nuclei were counterstained with fluorescent stain 4′,6-diamidino-2-phenylindole (DAPI) and slides were mounted with fluorosave reagent (Merck). The details of all the primary and secondary antibodies are listed in Table 2.

The details of primary antibodies used viz. BDNF, GDNF, CNTF, GFAP, Aβ42, and Caspase3 is mentioned which includes host, specificity, make, catalog no., and dilution.

Reactive Oxygen Species

Biochemical assay was done to estimate the levels of Reactive oxygen species. The hippocampus was dissected out from mouse brain and homogenized with 1×PBS. The homogenate was mixed with Dichlorodihydrofluorescein diacetate dye (DCFDA) from 3.05 mg stock prepared in 5 ml methane. By the action of cellular esterases and upon oxidation, it turns to fluorescent 2′,7′-Dichlorodihydrofluorescein. The readings were obtained in duplicates. The fluorescent intensity was measured at 488-nm excitation and 525-nm emission filters using Fluorimetry (BiotecK).

Statistical Analysis

The results were represented as mean ± S.E.M. Data was analyzed using GraphPad Prism 7.04. The normality of data was checked using 1-KS sampling. In MWM, the acquisition and retrieval data was analyzed by two-way ANOVA. Further, Tukey's test was used for post-hoc analysis in order to compare mean between days and groups. For passive avoidance and real-time PCR, data was analyzed by one-way ANOVA with Tukey's multiple comparisons test for post-hoc. For amyloid fluorescent intensity analysis, student *t* test was applied whereas for all the marker analysis by IHC and real-time PCR, one-way ANOVA with Tukey's test was applied. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$ were regarded as statistically significant.

Results

Aβ-42-induced Spatial Memory Loss Was Ameliorated by Transplantation of hUCB-derived Lin-ve Stem Cells

We used the Morris water maze (MWM) data from our recently published article (with permission from Bali et al. 2017) [13] in order to compare the effects of TrkB inhibitor on the effect of Lin-ve stem cell transplantation in amyloid β-42 injured mice. The injected oligomeric form of 1uM amyloid β-42 in the hippocampal region resulted in memory loss in Aβ-injected mice, as assessed by escape latency time (ELT) in MWM. ELT was significantly higher in amyloid β-42-injured mice when compared to sham control. The injured mice when transplanted with 50,000 Lin-ve stem cells, showing significant improvement in day-wise (acquisition days 1–6) escape latency and found to be comparable to the sham control mice. In our previous study, we have shown the effect of lineage negative stem cells after 10 and 60 days with cell doses of

Table 2 List of details of primary and secondary antibodies

Sr. no.	Primary antibodies	Specificity	Make	Catalog no.	Dilution
1.	BDNF	Mouse monoclonal IgG1	Santa Cruz Biotechnology	sc-65514	1:100
2.	GDNF	Mouse monoclonal IgG1 (kappa light chain)	Santa Cruz Biotechnology	sc-13147	1:100
3.	CNTF	Rabbit polyclonal IgG	Santa Cruz Biotechnology	sc-13996	1:100
4.	GFAP	Polyclonal rabbit anti-mouse	Sigma	HPA056030-100UL	1:100
5.	Aβ42	Rabbit polyclonal mouse	Elabscience	E-AB 40038	1:100
6.	Caspase3	Mouse monoclonal IgG1	Santa Cruz Biotechnology	sc-271759	1:100

50,000 as well as 100,000 [15]. We had described a dose-dependent recovery of cognitive impairment in amyloid-injured mice using 50,000 cells at 60 day. In our comparative analysis, results showed effective rescue by 50,000 Lin-ve stem cells when used at 10-day time-point with 1 μM concentration induced Aβ injury [13]. Hence, we used this dose and time-point for further investigation.

This improvement in ELT in the hUCB stem cell transplanted mice was noticed after they were injected with TrkB inhibitor (ANA-12) daily before acquisition and retrieval trials (Fig. 2a). On retrieval day (7th day), the time spent in each quadrant was measured. The amyloid-injected mice did not spend more time in the target quadrant (Q1) as seen in the sham control group. However, the time spent by the mice with Lin-ve stem cell transplantation was marginally increased when compared to amyloid injury mice. The inhibitory effect of TrkB on the retrieval trials was found to be comparable to amyloid injury mice injected with ANA-12 (Fig. 2b). On retrieval day (day 7), the mean distance from the platform was calculated and found increased significantly in mice injected with amyloid β when compared to control groups. The distance was also found to be reduced in mice transplanted with stem cells. Although insignificant, this distance was further increased in ANA-12-injected mice, suggesting the crucial role of TrkB signaling in the effect of Lin-ve stem cell-mediated cognitive improvement in the amyloid β injured mice (Fig. 2c).

Improvement of Fear Conditioning Memory after hUCB Stem Cell Transplantation

To further confirm the learning and memory quotient in mice of various groups, we performed another behavioral test, i.e., passive avoidance. The learning and memory is estimated by the time taken by mice to avoid aversive stimuli (i.e., electric shock). Memory is positively correlated with the time taken by mice to move from light to dark chamber. The increase in latency signifies improved retrieval of fear-associated memory. The results showed significant reduction in latency time in amyloid-injected mice when compared to healthy control ($p \leq 0.0001$), suggesting worsening of associative memory in amyloid-injected group. The intra-hippocampal transplantation of human Lin-ve stem cells resulted in significant increase ($p < 0.01$) in latency as compared to the amyloid injury group. This improvement in latency was found to be reversed in the mice injected with TrkB inhibitor ($p < 0.001$) (Fig. 2d).

Swimming Track Plots Depict Behavioral Pattern

During MWM experiment, all the trials were analyzed using Anymaze software in order to analyze swimming pattern of mice. The track plots from acquisition days (days 1–6) were assessed for sham control, amyloid injury, stem cell transplantation, and TrkB inhibitor-injected mice. The reduction in swimming path and movement around the platform was observed in mice of sham control groups as the acquisition days progressed. Amyloid injury group showed circular movement around the periphery of tank with complex swimming track pattern. However, the hUCB Lin-ve cell transplanted mice showed better swimming performance and concomitant reduction in path, unlike injured mice. This improvement was further reversed when the mice were injected with TrkB inhibitor, showing similar pattern of swimming track as of amyloid injured mice (Fig. 3).

hUCB Lin-ve Stem Cell Transplantation Reduces Amyloid Load in Mouse Brain

The improvement in behavioral tests was found associated with amyloid staining in the mouse brains transplanted with Lin-ve stem cells. The immunohistochemical analysis for Cy3-Aβ antibody revealed deposition of amyloid aggregates around the site of injection (Fig. 4a). These aggregates were found to be significantly reduced in the mice brains after 20 days of hUCB Lin-ve stem cell transplantation (Fig. 4b). The fluorescent intensity measured by ImageJ showed significant reduction of corrected total cell fluorescence (CTCF) in the hUCB Lin-ve stem cell-transplanted brain sections as compared to amyloid injured brains (Fig. 4c).

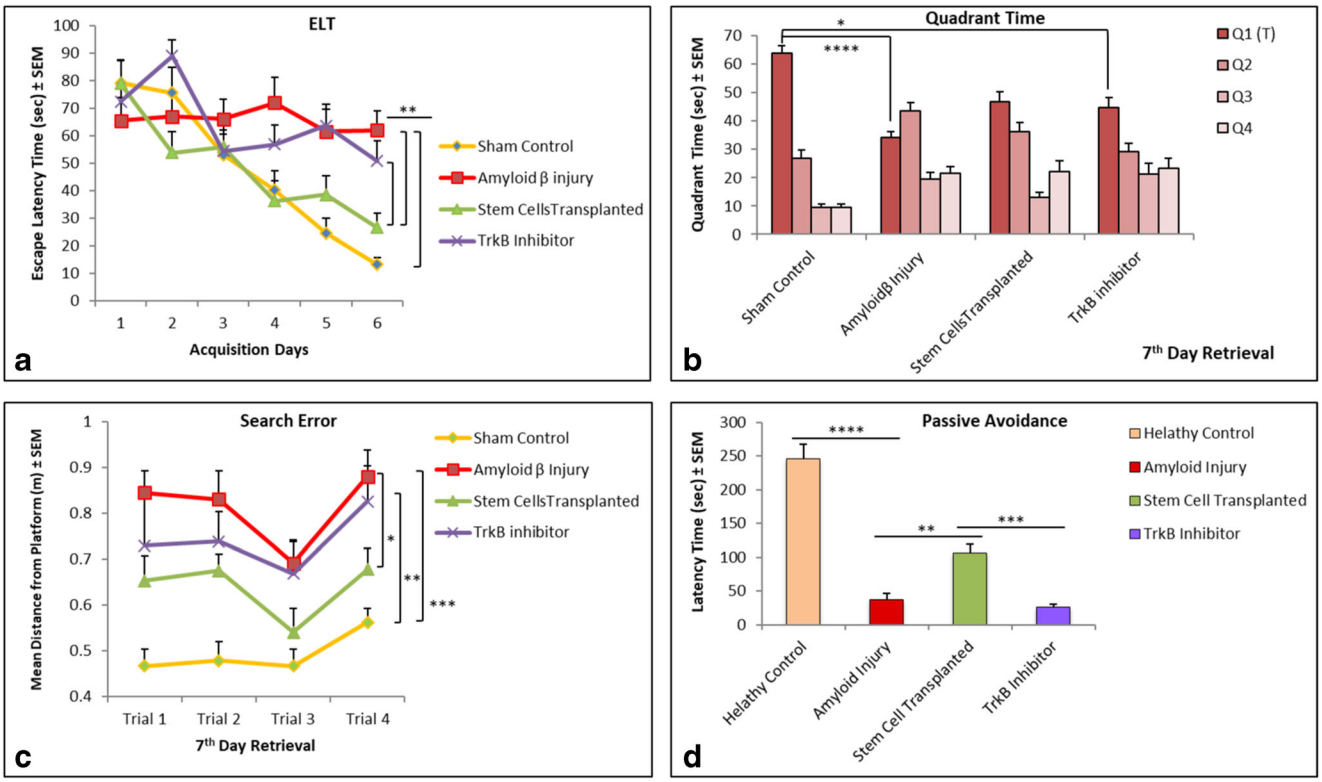

Fig. 2 Neurobehavioral analysis suggesting involvement of TrkB pathway in amelioration of memory loss by Lin-ve stem cells. **a** The graph depicts day-wise escape latency time during acquisition days of mice in sham control ($N = 7$), amyloid β injury ($N = 7$), stem cell transplantation ($N = 8$), and TrkB inhibitor ($N = 10$). The ELT was found to be more in mice with Aβ injury as compared to the sham control group whereas; stem cell transplantation ameliorated the memory loss depicted by reduced ELT. The mice were administered with TrkB inhibitor resulted in significant increase of ELT along the acquisition days. **b** The graph showing time spent by mice of different groups in MWM quadrants Q1, Q2, Q3, and Q4 on retrieval day (7th day). Mice with Aβ injury spent less time in target quadrant (Q1) in comparison to sham control and stem cell-transplanted group. **c** The search error graph showing mean distance from hidden platform traveled by mice. The mice with Aβ injury traveled at significantly more distant from the platform in comparison to sham control and stem cell-transplanted groups. The mice administered with TrkB inhibitor after stem cell transplantation in mice with Aβ injury resulted in more mean distance from platform comparable to Aβ injury group. **d** Passive avoidance graph depicts the role of TrkB pathway in amelioration of memory loss. The stem cell transplanted with Aβ injury mice showed significant increase in latency time (in sec). The administration of TrkB inhibitor (ANA12) significantly reduced the latency time suggesting involvement of TrkB pathway in rescue of memory loss. For MWM, data was analyzed by 2-way ANOVA with acquisition days and retrieval trials as a measure of repeated observations. Further, Tukey's test was used for post-hoc analysis to compare mean between days and groups. For passive avoidance, one-way ANOVA with Tukey's multiple comparisons test for post-hoc was applied. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ were regarded as statistically significant

hUCB Lin-ve Stem Cells Modulate Neurotrophic Secretion

The mRNA and protein expression of neurotrophic factors was analyzed by real time PCR and immunohistochemistry, respectively. We report significant reduction of BDNF expression in mRNA as well as protein levels in the amyloid injury group when compared to controls whereas hUCB Lin-ve stem cell transplantation upregulated its corresponding protein expression. Although TrkB inhibitor could not alter the mRNA levels in the mice brain (Fig. 5a–e), the expression of GDNF mRNA and protein was found to be reduced in the amyloid injury group and after transplantation of hUCB Lin-ve stem cells, it was significantly increased as compared to injured brain. Further, GDNF mRNA expression was significantly reduced when mice were injected with ANA-12 (TrkB antagonist) (Fig. 5f–j). Although non-significant, CNTF expression was also found to be increased after transplantation of hUCB Lin-ve stem cells when compared to amyloid-injured brains. It remained undetermined while the endogenous expression of CNTF was very low in healthy control mice brains. This expression was further abolished in mice in which ANA-12 was administered (Fig. 5k–o). Glial fibrillary acidic protein (GFAP) expression was also found to be significantly decreased in amyloid injury brains and when transplanted with hUCB Lin-ve stem cells, their expression was significantly upregulated. TrkB inhibitor did not alter mRNA expression of this protein when compared to either amyloid injury or hUCB Lin-ve stem cell-transplanted brains (Fig. 5p–t).

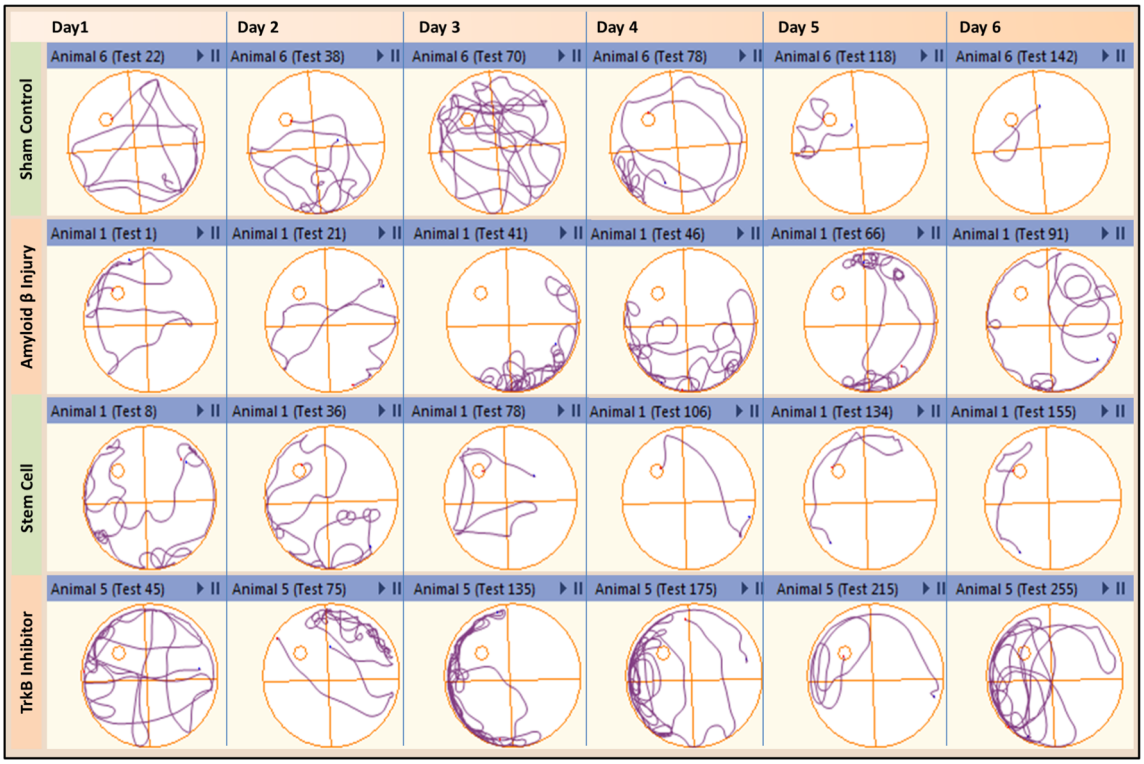

Fig. 3 MWM track plot representative swimming track plots as an index of learning from acquisition days 2, 4, 5, and 6 of different groups. The track plots depict that Aβ injury mice could not reduce their swimming path and moved towards periphery of MWM tank. The stem cell transplantation in Aβ injury mice resulted in shortening of path and movement more towards the hidden platform along the acquisition days, which is comparable to sham control group. The TrkB-administered mice could not reduce their path and moved more towards periphery region depicting memory loss

hUCB Lin-ve Stem Cells Prevent Cellular Apoptosis and Enhances Proliferation

The amyloid β is known to induce series of apoptotic cascade which eventually leads to neuronal cell death. We wanted to estimate the levels of apoptotic and proliferative markers in brains after hUCB Lin-ve stem cell transplantation in order to evaluate the mechanism through which these cells exert therapeutic effects in amyloid-injured mice brains. The protein level of Caspase3, an apoptotic marker, was found to be significantly upregulated in amyloid injury brains. This was found to be ameliorated by hUCB Lin-ve stem cell transplantation at the site of injury. The mRNA levels of Bcl-2, an anti-apoptotic marker (Fig. 6a–c) were significantly reduced by amyloid β aggregation in comparison to healthy controls. This reversed significantly after the transplantation of hUCB Lin-ve stem cells. This increase in expression was blunted by TrkB inhibitor in these brains (Fig. 6d). Further, Ki-67, a marker for cellular proliferation [41] expressed during the active phases of cell cycle [42], was found to be significantly reduced in amyloid injury group in comparison to control brains. This was partly rescued by hUCB Lin-ve stem cell transplantation. This induced Ki-67 expression was reversed when mice were administered with ANA-12 (Fig. 6e).

The levels of hydroxyl, peroxyl, and other reactive oxygen species (ROS) activity were also assessed using 2′,7′–dichlorofluorescindiacetate (DCFDA) in hippocampus. The levels of ROS were measured by the fluorescent intensity of 2′,7′–dichlorofluorescein (DCF) compound released from DCFDA by the action of cellular esterase. Our data suggests that there were no changes in the ROS activity in the hippocampus from all the groups (Fig. 6f).

Discussion

Our data provides the fundamental framework for therapeutic efficacy of hUCB-derived Lin-ve stem cells in the mouse model of Aβ-42 injury. The transplantation of hUCB-derived Lin-ve stem cells after amyloid injury resulted in decrease of day-wise escape latency time in MWM test, comparable to control groups (Fig. 2). Similarly, in track plot analysis, the mice with transplanted Lin-ve stem cells took lesser time to locate the platform in comparison to Aβ-42 injury Lin-ve mice (Fig. 3). Hence, shortening of swimming path suggests that hUCB Lin-ve stem cells have potential to rescue the spatial memory loss induced by Aβ-42. We also subjected these mice to passive avoidance analysis in which mice

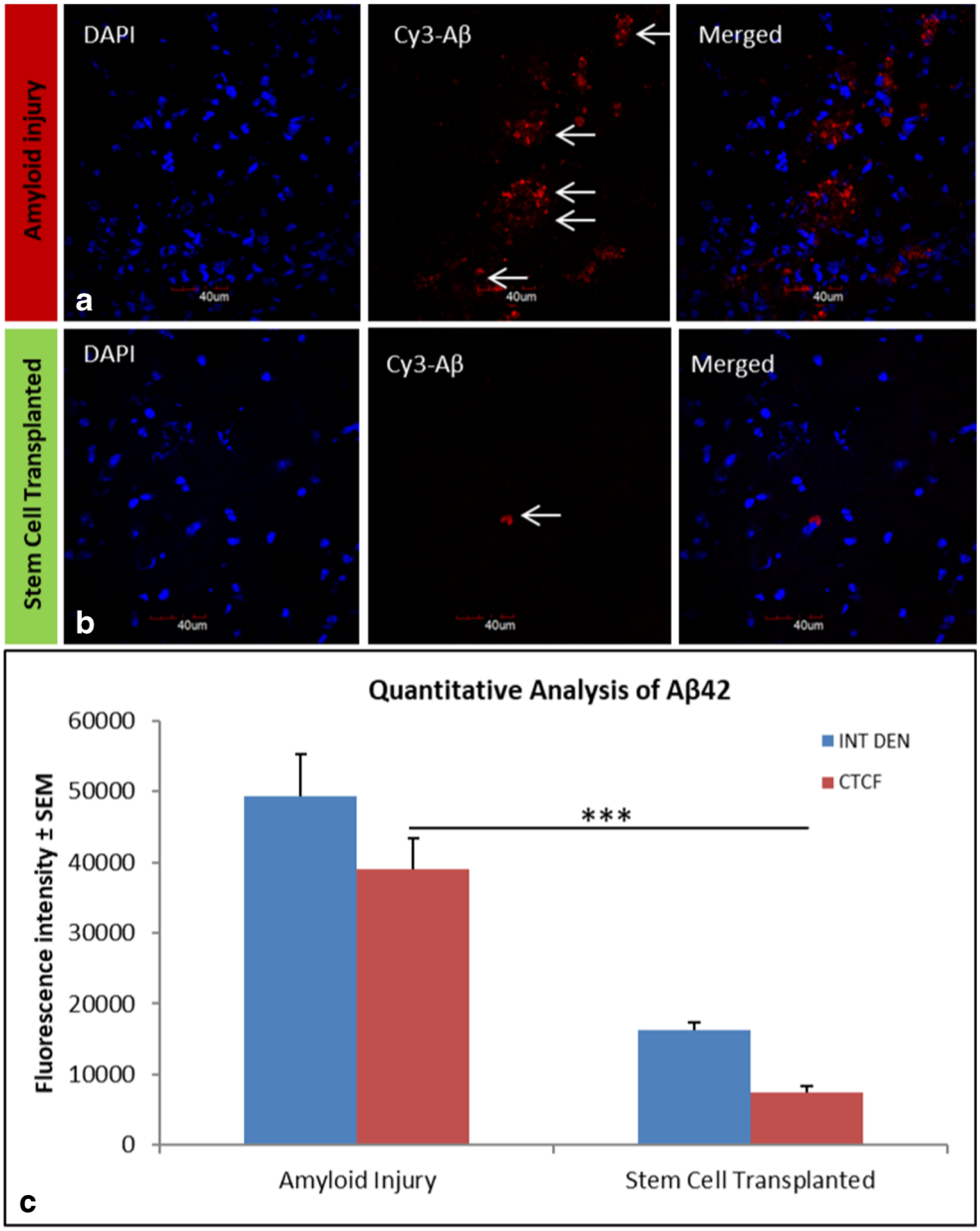

Fig. 4 Amyloid deposition and its clearance by Lin-ve stem cells. **a** Immunohistochemistry analysis using primary antibody (1:100 dilutions) of Aβ-42 and secondary antibody (1:200 dilutions) showed deposition of amyloid aggregates near hippocampal region in Aβ injury group at × 10 and × 60 magnification. However, we found significant reduction in amyloid aggregates in mice transplanted with Lin-ve stem cells as visualized under confocal microscope at × 20 and × 60 magnification. **b** Quantification of the hippocampal brain sections based on florescence intensity using ImageJ software Data was statistically analyzed using GraphPad Prism 7.04. Student *t* test was applied between the groups. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ were regarded as statistically significant

transplanted with hUCB Lin-ve stem cells showed better memory retrieval with increased latency to stimuli (Fig. 2).

Our previous study had shown that this improvement is mediated by the upregulation of two putative neurotrophic factors, i.e., BDNF and CREB (cAMP response element-binding) [15]. It is also well known that BDNF binds to tropomyosin receptor kinase B (TrkB) which dimerizes to initiate three pathways, i.e., PKC, PI3, and Ras/MAPK pathways [43]. These pathways eventually lead to the activation of transcription factor CREB which further activates the genes involved in synaptic plasticity [43]. Therefore, we targeted the TrkB pathway using ANA12, a non-competitive antagonist of TrkB receptor, because it has been shown to successfully block the action of BDNF [44]. The mice administrated with ANA12 showed reversal of behavioral outcomes by hUCB Lin-ve stem cell transplantation, as confirmed by both MWM (Fig. 3a–c) and passive avoidance test (Fig. 3d). This provides a compelling argument to suggest that the hUCB Lin-ve stem cells rescue memory loss via BDNF-TrkB pathway.

We further sought to determine if the hUCB Lin-ve stem cells could reduce the artificially deposited amyloid load from the mouse brain. In order to evaluate this, we examined the deposits of Aβ-42 in the hippocampus by immunohistochemistry. We found significant reduction of Aβ deposits in hUCB Lin-ve stem cell-transplanted group as compared to the amyloid injury group (Fig. 4). We found that the clearance of Aβ from the brain was associated with activation of astrocytes

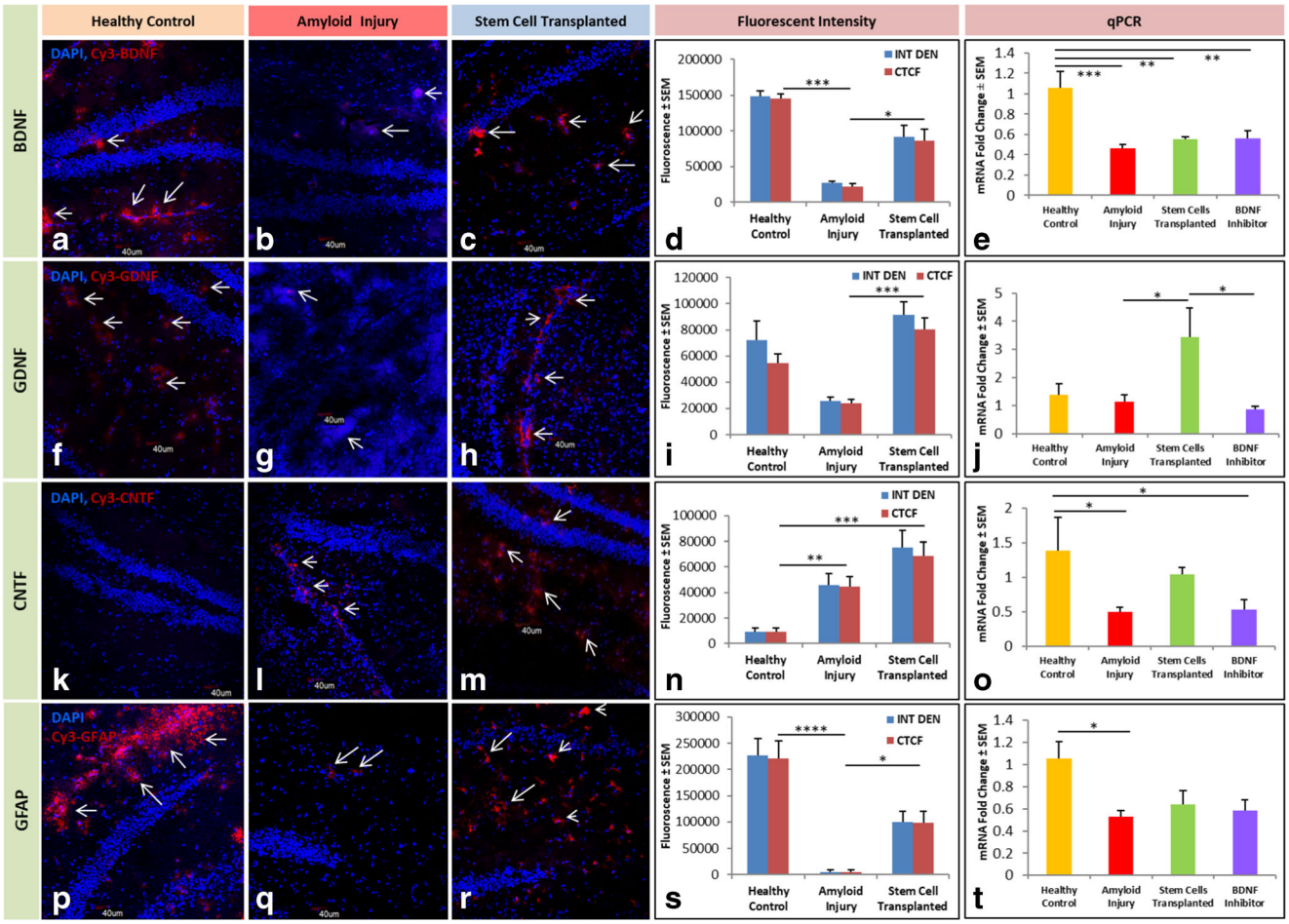

Fig. 5 Modulation of neurotrophic factors and activation of astrocytes by Lin-ve SC. **a**–**c** Immunohistochemistry showing cy3 expression (red) bound to primary antibody specific for BDNF at 20× in healthy control, amyloid injury and stem cell-transplanted group and counterstained with DAPI. **d** BDNF IHC images were quantified based on florescence intensity using ImageJ software. **e** The mRNA expression of BDNF analyzed by real-time PCR showed significant reduction in amyloid injury group ($p = 0.0005$) as well as in the TrkB inhibitor group ($p = 0.0017$) in comparison to healthy control. **f**–**h** GDNF expression (cy3-red) at 20× was analyzed in immunohistochemistry using coronal section of hippocampus merged with DAPI staining. **i** GDNF IHC images were quantified based on florescence intensity using ImageJ software. **j** The mRNA expression of GDNF analyzed by real-time PCR showed significant increase in stem cell-transplanted group in comparison to amyloid injury group ($p = 0.0447$). Also, expression was significantly reduced after administration of TrkB inhibitor (ANA12) ($p = 0.0207$). **k**–**m** Similarly, CNTF expression was analyzed using immunohistochemistry in all three groups at 20×. **n** IHC of CNTF images were quantified based on florescence intensity using ImageJ software. **o** The mRNA expression of CNTF analyzed by real-time PCR showed significant reduction in amyloid injury group ($p = 0.0405$) as well as in the TrkB inhibitor group ($p = 0.0485$) in comparison to healthy control. **p**–**r** GFAP expression was similarly visualized in immunohistochemistry merged with DAPI at 20×. **s** Quantification of the hippocampal brain sections based on florescence intensity using ImageJ software. All sections were analyzed under confocal microscope. **t** mRNA expression of GFAP was found to be significantly reduced by Aβ injury as compared to the healthy control group ($p = 0.0139$). For all the marker analysis by IHC and real-time PCR, one-way ANOVA with Tukey for post-hoc analysis was applied. $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$ were regarded as statistically significant

(increased level of GFAP), as suggestive from previous data showing astrocytes' role in both Aβ clearance as well as in modulation of neuroinflammation [45]. The astrocytes take up the Aβ-ApoE complexes from the extracellular matrix and degrade by neprilysin, insulin-degrading enzyme, or matrix metalloproteinase-9 (MMP-9) (Wyss-Coray). The accumulation of Aβ is often suggestive of the failure of its clearance by astrocytes, resulting in astroglial pathology. We showed that the Aβ-induced injury group showed significant reduction in its mRNA expression besides corresponding changes in the protein expression. The astrocyte expression after hUCB Lin-ve stem cell transplantation was found significantly increased when analyzed by immunohistochemistry of mouse hippocampus; however, no change was observed in the associated mRNA expression (Fig. 5p–t). These results indicate that hUCB Lin-ve stem cells facilitate scavenging of Aβ by the activation of hippocampal astrocytes.

As Aβ is the major contributor for amyloid plaque formation, it initiates the pathological events inducing formation of neurofibrillary tangles (NFT) and neuronal cell death [46, 47].

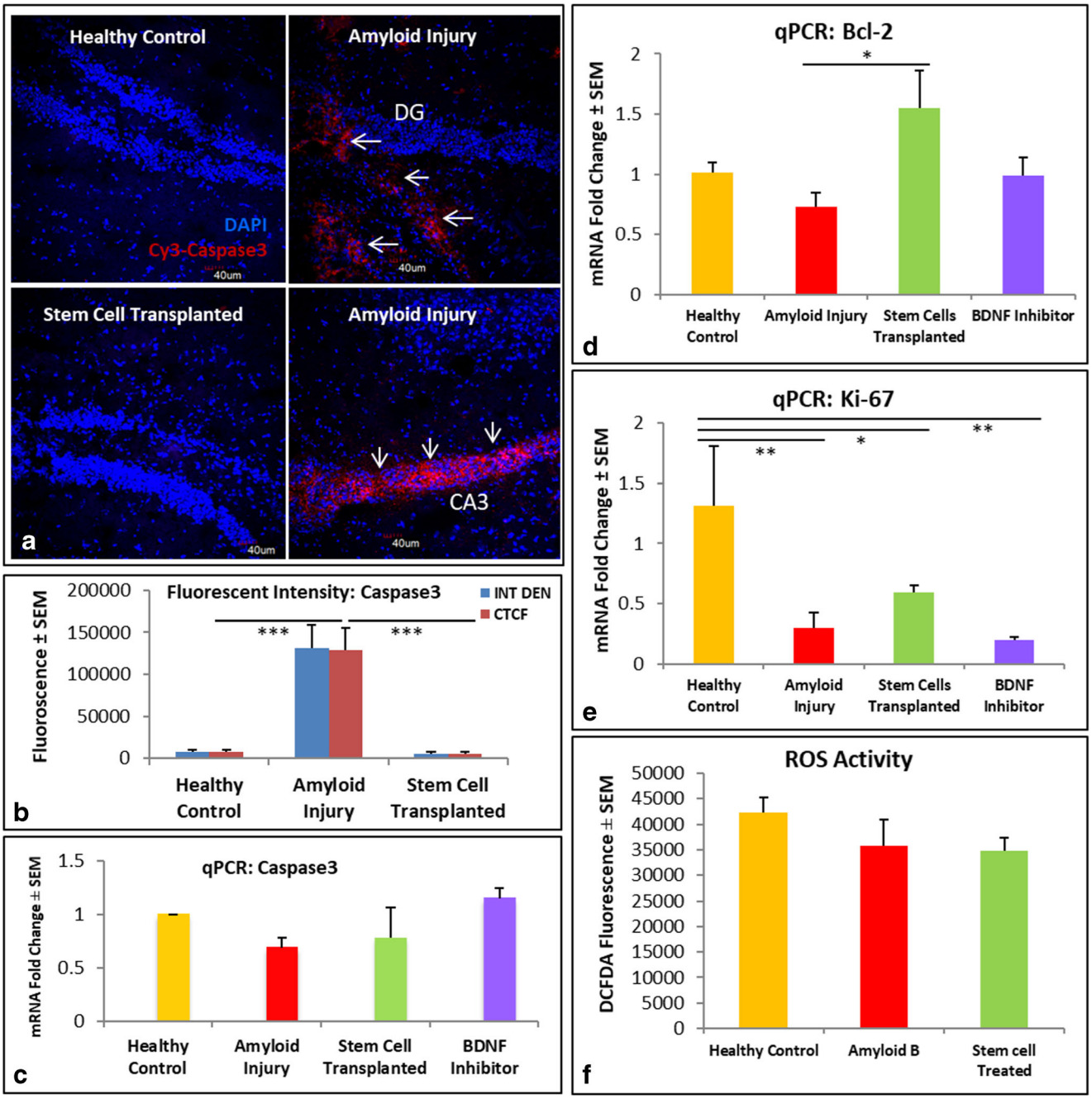

Fig. 6 Lin-ve SC exerts anti-apoptotic activity. **a** Immunohistochemistry of caspase3 was performed in mouse brain hippocampal sections of healthy control, Aβ injury, and stem cell-transplanted group. The upregulated expression of caspase3 in Aβ injury mice was observed in hippocampus especially dentate gyrus region as well as in CA3 region and reduced in stem cell-transplanted group. **b** Caspase3 IHC images were quantified based on florescence intensity using ImageJ software. **c** mRNA expression of Caspase3 was found to be changed non-significantly analyzed by qPCR. **d** mRNA expression of Bcl2 was found to be significantly higher in stem cell-transplanted group in comparison to Aβ injury group analyzed by real-time PCR ($p = 0.0245$). **e** mRNA expression of Ki67 was significantly reduced by the Aβ injury group compared to healthy control ($p = 0.0026$), however, was found to be increased in stem cell-transplanted group compared to injury group, but non-significant ($p = 0.5135$). TrkB inhibitor-injected group showed significant reduction of Ki-67 compared to healthy control ($p = 0.001$). **f** The reactive oxygen species levels was analyzed using DCFDA fluorogenic dye and levels were estimated in healthy control, Aβ injury, and stem cell-transplanted group using fluorimeter. There was no difference recorded among the groups. One-way ANOVA with Tukey for post-hoc analysis was applied. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ were regarded as statistically significant

The Aβ-associated apoptosis studies have already uncovered the underlying mechanisms [47]. It is for this reason that we wanted to analyze the effect of Aβ injection on neuronal cell death and consequent memory loss. Caspase3, which is a hallmark of apoptosis, was found to be significantly upregulated in the hippocampus (especially dentate gyrus and CA3

region), when analyzed by immunohistochemistry. However, apoptosis was significantly reduced when hUCB Lin-ve stem cells were transplanted. The corresponding mRNA expression was, however, not significant (Fig. 6a–c). Similarly, Bcl2, which is a marker for anti-apoptotic activity, was analyzed by real time PCR and found to be unaltered in the amyloid injury group, whereas a significant increase was noted after hUCB Lin-ve stem cell transplantation. The administration of TrkB pathway inhibitor showed comparable results as reminiscent of amyloid injury.

The oligomers of Aβ along with tau protein are considered as causative of neuroinflammation, cholinergic denervation, and synaptic loss via oxidative stress [48]. It is also widely believed that the mitochondrial dysfunction causes neurodegeneration in AD and is associated with ROS generation [49]. When ROS was analyzed in the Aβ-injected hippocampus and the hUCB Lin-ve stem cell transplantation group, no changes was noticed (Fig. 6f) indicating the dominant role of other processes described in this paper. Hence, it can be concluded that BDNF, Caspase3 and Bcl-2 play dominant role in the rescue of memory loss than ROS. In order to further probe whether increase in neurotrophic factors and reduction in apoptosis was associated with neuronal cell proliferation, we examined proliferating cell nuclear antigen, i.e., ki67, used to identify actively dividing cells. Expectedly, the ki67 was significantly downregulated in the amyloid injury group and marginally increased after hUCB Lin-ve stem cell transplantation (Fig. 6e). The expression of ki67 can be associated with the GDNF expression as it has a significant role in proliferation [50]. It is thus compelling to mention that Aβ-42-induced memory loss was prevented by transplantation of hUCB Lin-ve stem cell through modulation of neuroinflammation and apoptotic pathways.

Further, CNTF expression was also found to be significantly reduced in the injury group and increased in the hUCB Lin-ve stem cell-transplanted group. [51]. The most important downstream molecule of this pathway is Bcl-2 [52], a well-identified anti-apoptotic marker, which was found to be significantly upregulated in hUCB Lin-ve stem cell-transplanted mice brains. It can thus be speculated that hUCB Lin-ve stem cells induced amelioration of memory loss is partly due to activation of anti-apoptotic machinery via Bcl-2. It needs

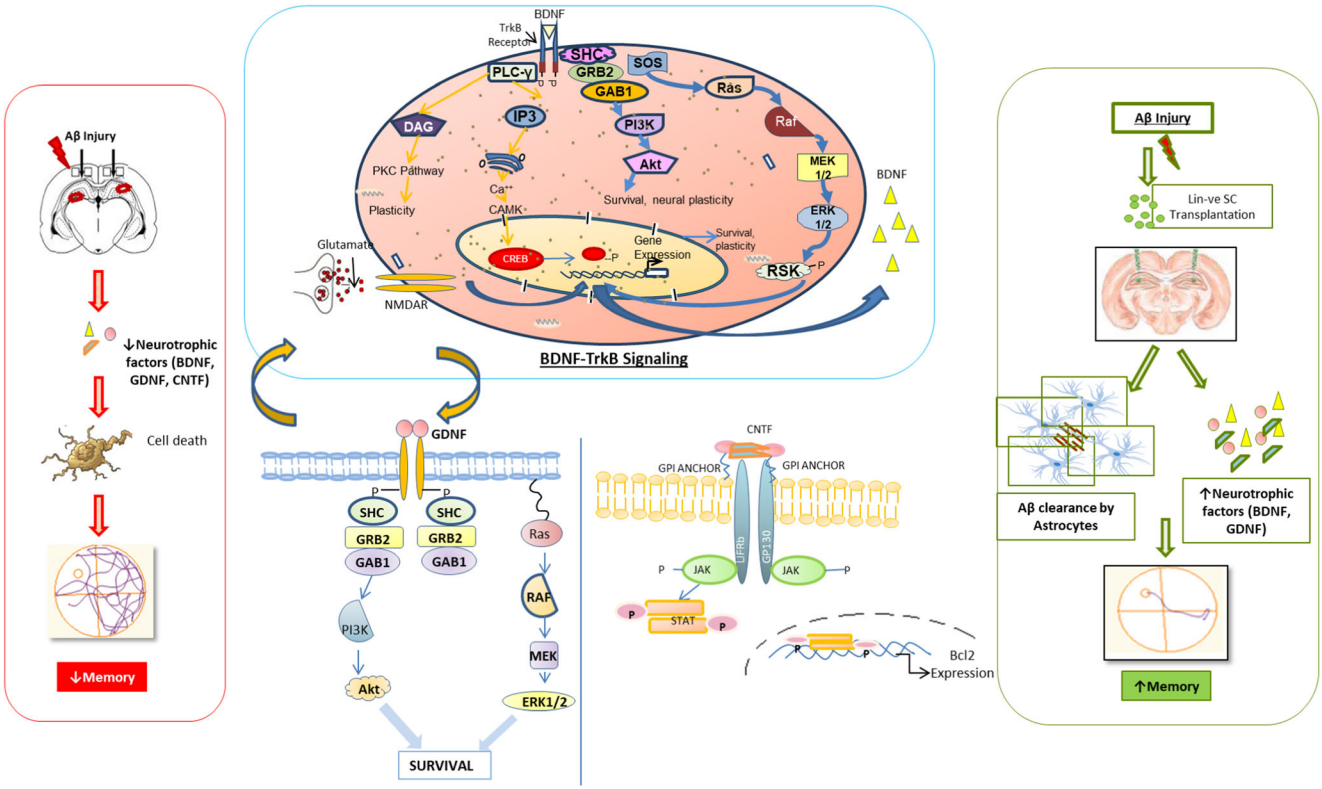

Fig. 7 Schematic of overall molecular mechanism initiated by Lin-ve stem cells. The schematic showing possible mechanisms involved by the transplantation of UCB derived Lin-ve stem cells. Our study showed that amyloid injury reduces neurotrophic factors which would affect neurons by cell death. The transplantation of Lin-ve exerted paracrine effects by modulating or increasing the production of neurotrophic factors, i.e., GDNF, BDNF, and CNTF. This might have activated astrocytes which possibly involved in the clearance of Aβ deposits and hence alleviating memory loss. The Lin-ve stem cells exerted its paracrine effects by the involvement of TrkB pathway. This study also suggested that there might be the involvement of RET pathway as indicated by upregulation of GDNF and cross-talk which exists with TrkB pathway [26]. Similarly, upregulation of CNTF and Bcl2 also indicated the probable role of Jak-STAT pathway mediated by these transplanted cells bringing out the therapeutic outcome in this study. Hence, our study suggests that Lin-ve stem cells have the potential to ameliorate cognitive impairment by initiating complex molecular mechanisms by its paracrine effects

further investigation to determine if it is directly mediated by CNTF. However, a study has previously linked the role of CNTF in the activation of astrocytes [53]. In order to test whether hUCB Lin-ve stem cells exerted paracrine effects via the CNTF secretion, mediated by astrocytes or anti-apoptotic activity via JAK-STAT pathway, additional experiments are imperative.

GDNF is another putative neurotrophic factor, which is also implicated in AD and was decreased in MTG of AD postmortem brains [23]. Its overexpression has been also shown to improve the learning and memory by concomitant upregulation of BDNF. Our results showed decrease in the mRNA expression of GDNF in the amyloid injury group whereas there was significant increase in the hUCB Lin-ve stem cell-transplanted group. This suggests that GDNF plays active role in reversal of memory loss by hUCB Lin-ve stem cell transplantation. Furthermore, the mRNA expression of GDNF was significantly reduced after ANA12 administration in comparison to the hUCB Lin-ve stem cell-transplanted group. This suggests that BDNF-TrkB pathway may be associated with GDNF expression. The therapeutic outcome in our study could be possibly due to the cross-talk between BDNF induced TrkB signaling and GDNF induced RET pathway (15).

ANA-12 is a non-competitive antagonist of TrkB receptor, chiefly blocking the action of BDNF [44]. ANA-12 was administered intra-peritoneally before behavioral performance. The mice administered with TrkB receptor inhibitor resulted in significant increase in escape latency time as well as the search error, i.e., mean distance from platform, which was found to be comparable to amyloid injury mice (Fig. 2a–c). This was further confirmed by fear conditioning memory using passive avoidance test. On test day, the latency to cross from light to dark compartment was significantly increased in Lin-ve stem cell-transplanted group compared to the amyloid injury group (Fig. 2d). By inhibition of TrkB receptors, the complete reversal of the neurobehavioral outcomes from transplanted hUCB Lin-ve stem cells was noted. This data suggests that hUCB Lin-ve stem cells could effectively rescue memory loss via TrkB signaling.

It can be further speculated whether the restoration of neurotrophic factors such as BDNF, GDNF, and CNTF in our study is released by transplanted Lin-ve stem cells or these cells stimulated endogenous neurons or astrocytes to secret these factors? We analyzed the expression of these neurotrophic factors using mouse primers as well as antibodies utilizing real-time PCR and immunohistochemistry respectively. The source of BDNF is from endogenous neurons; GDNF is secreted by glial cells while CNTF is secreted by astrocytes. This can be further tested using co-culture of stem cells with neurons/astrocytes. Recent study by Joseph Park et al. developed unique 3D triculture system, which is found to be effective for conducting such study of co-culture of stem cells with neurons/astrocytes, in order to decipher the paracrine role of Lin-ve stem cells on neurons/astrocytes. This model has successfully recapitulated AD pathology because it mimics the in vivo extracellular matrix as in AD brains and 3D environment provides a large surface area for growth and differentiation of neurons/astrocytes [54].

As Lin-ve stem cells mediate its effect via release of neurotrophic factors, therefore, we further carried out the comparative analysis by injecting BDNF and compared it with Lin-ve cell transplanted group (data not published). This study is currently ongoing and further experiments are required before we test the hypothesis of BDNF-mediated effects. Although, the aim of the paper was to develop understanding of the effect of Lin-ve stem cells transplantation on neurotrophic factors, future studies can also examine the role of diet, circadian rhythmicity, gender, etc. on stem cell-mediated reversal of memory loss.

Conclusions

Our results reveal that transplantation of hUCB Lin-ve stem cell can rescue the memory loss by clearance of Aβ mediated by activation of astrocytes. hUCB Lin-ve stem cells exert paracrine effects by modulating the hippocampus neurochemistry as noted by escalation of neurotrophic factors. The increase in neurotrophic factors by hUCB Lin-ve stem cell transplantation may also exert anti-apoptotic effects and may activate complex molecular pathways involving TrkB, RET and Jak-STAT (Fig. 7). It will be of interest to dissect the putative molecular pathway in future studies for further insights.

Acknowledgements We thank Prof. Jaswinder Kalra for providing samples of umbilical cord blood from CLROT of PGIMER. We also thank Sridhar Bammidi for training the first author in some techniques described in the manuscript and Mr. Gurpreet Singh for imaging.

Author Contribution PB conducted all the experiments, acquisition of the data, and writing of manuscript. AB was involved in manuscript writing/editing and data/statistical analysis. BN was first author's PhD supervisor and edited the manuscript. AA conceptualized the study, secured research grant, and edited the manuscript.

Source of Funding Department of Biotechnology, New Delhi, India and Council of Scientific & Industrial Research (CSIR), India.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations Lin-ve, Lineage negative; SC, Stem cells; UCB, Umbilical cord blood; BDNF, Brain-derived neurotrophic factor; GDNF, Glial-derived neurotrophic factor; CNTF, Ciliary neurotrophic factor; TrkB, Tyrosine receptor kinase B; Bcl2, B cell lymphoma 2;

JAK, Janus kinases (JAKs); STAT, Signal transducer and activator of transcription proteins; DG, Dentate gyrus; CA, Cornu Ammonis; Aβ, Amyloid β

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