

Development of Neural Stem Cell Targeted Cell Derived Surface Engineered Extracellular Vesicle for Adult Neurogenesis

2.1 ABSTRACT

Aging and various neurodegenerative diseases cause significant reduction in adult neurogenesis and simultaneously increase in quiescent neural stem cells (NSC), which impact the brain's regenerative capabilities. To deal with this challenging issue, current treatments involve stem cell transplants or prevention of neurodegeneration; however, the efficacy or success of this process remains limited. Therefore, extensive and focused investigation is highly demanding to overcome this challenging task. Here, we have designed an efficient peptide-based EphA4 receptor-targeted ligand through an in-silico approach. Further, this strategy involves chemical conjugation of the peptide with adipose tissue stem cell-derived EV (Exo-pep-11). Interestingly, our newly designed engineered EV, Exo-pep-11, targets NSC through EphA4 receptors, which offers promising therapeutic advantages by stimulating NSC proliferation and subsequent differentiation. Our result demonstrates that NSC successfully internalized Exo-pep-11 in both *in vitro* culture conditions as well as *in vivo* aging rats. We found that the uptake of Exo-pep-11 decreased by ~2.3 fold when NSC was treated with EphA4 antibody before Exo-pep-11 incubation, which confirms the receptor-specific uptake of Exo-pep-11. Exo-pep-11 treatment also increases NSC proliferation by ~1.9 fold and also shows ~1.4 fold and ~2.4-fold increase in expression of Nestin and ID1, respectively. Exo-pep-11 also has the potential to increase neurogenesis in the aging rat, which is confirmed by ~1.6-fold and ~1.5-fold increase in expression of TH and Tuj1, respectively in rat Olfactory Bulb. Overall, our findings highlight the potential role of Exo-pep-11 for prospective applications in combating age-related decline in NSC activity and neurogenesis.

2.2 INTRODUCTION

Adult neurogenesis refers to the intricate process wherein neural stem cells (NSC) located in distinct brain regions, namely the subventricular zone (SVZ) and the Subgranular Zone (SGZ), give rise to new neurons. Specifically, the SVZ contributes interneurons to the olfactory bulb, while the SGZ contributes neurons to the hippocampal dentate gyrus.^{1, 2} The proliferation of neural stem cell (NSC) within the subventricular zone (SVZ) occurs along the medial wall of the lateral ventricle (LV). Stem cells undergo division, producing transit-amplifying cells. These amplifying cells, in turn, give rise to neuroblasts that embark on a migratory journey through the rostral migratory stream (RMS), reaching their ultimate destination in the olfactory bulb (O.B.).³ Every day, more than 30,000 neuroblasts emerge from the mouse subventricular zone (SVZ), journeying anteriorly. They cover distances of up to 5 mm in rodents and 2 cm in primates, propelled at speeds of approximately 122 mm/h towards the olfactory bulb⁴⁻⁸. Upon reaching the olfactory bulb (O.B.), neuroblasts unite within the granule or periglomerular cell layer. Within this region, neuroblasts undergo maturation, developing into two primary classes of interneurons granular cells and periglomerular cells, thus becoming integral components of the olfactory bulb circuitry^{9, 10}.

The aging process has a detrimental impact on neurogenesis, leading to a pronounced and ongoing reduction in cell production within both the subventricular zone (SVZ) and the neurogenic niches of the hippocampus in the brain¹¹⁻¹³. As individuals undergo aging, adult neural stem cells (aNSCs) experience a decline in their ability to proliferate and enter a quiescent state¹⁴. Early studies in rodents revealed clear evidence of a decline in neural stem cell (NSC) proliferation and neurogenesis associated with aging¹⁵⁻¹⁸. For instance, commencing from the initial month of life, the rate of hippocampal neurogenesis decreases by approximately 40% each month during the first nine months in the mouse brain¹¹. These findings, along with other data, imply that the onset of neurogenesis decline occurs by middle age¹⁹⁻²¹. In aged animals, the decline in the population of newly generated neurons corresponds significantly with a substantial reduction in proliferation, typically occurring between 20 to 24 months of age, within both neural stem cell (NSC) niches^{19,22-24}. This decline appears to be selective, affecting neurogenic populations specifically, while other niche cells like SVZ astrocytes and ependymal cells remain unaffected and intact in aged animals²⁵. Therefore, the depletion of the functional neural stem cell pool is likely a driving factor in the overall decline of neurogenesis, potentially contributing to cognitive decline and the onset of neurodegenerative disorders in aged animals²⁶.

Extracellular Vesicles (EVs) are nanosized vesicles secreted by all cell types, playing crucial roles in cell-cell communication. They can act as disease pathogenic agents and are extensively investigated for the development of neurotherapeutics, leveraging their unique properties such as the ability to traverse the blood-brain barrier, non-invasiveness, non-tumorigenicity, non-immunogenicity, and their inability to replicate or induce microvascular embolism^{27,28}. EV has already been studied in the differentiation of neural stem or neural progenitor cell to neuron. Lee and colleagues showed that introducing miRNAs miR-124 and miR-145 into mesenchymal stem cells (MSCs) derived from extracellular vesicles (EVs) enhanced the differentiation of human neural progenitor cells (NPCs) to neural cells and boosted the expression of glutamate transporters⁵³. EVs derived from MSCs have been investigated for their potential in developing therapeutics targeting a range of conditions, including tumours, neurodegenerative diseases, cardiovascular and cerebrovascular diseases, as well as wound repair^{29-33,54-56}. To date, only one peptide has been identified with the capability to target neural stem cells and influence their properties³⁴.

EphA receptors predominantly bind to ephrin-A ligands, comprising a family of five glycosylphosphatidylinositol (GPI)-anchored proteins. An exception to this pattern is EphA4, which specifically binds to ephrin-B2 and B3 ligands³⁵. Experimental evidence has shown EphA4 is expressed only by neural stem cells (NSC)^{36,37}. EphA4 can be utilized to target NSC for targeted drug delivery, which will reduce off-targeting complicity.

The field of EV-mediated NSC targeting and development of neurotherapeutics has largely remained unexplored; towards this venture, we have designed an EphA4 targeting peptide based on the EphA4-EphrinB2 binding. This designed peptide was conjugated with EV, termed as Exo-Pep-11, and explored its effect in NSC targeting and therapeutic potential in aging rats utilizing SVZ-olfactory bulb neurogenesis as a model (Figure 2.1). Firstly, we have designed a library of probable EphA4-targeted peptides from the EphA4-EphrinB2 complex using alanine scanning and docking. The library was then screened using M.D. simulation, and we identified one lead peptide for surface functionalization of rat adipose tissue stem cell-derived EV. The surface-engineered EV, Exo-Pep-11, was then checked for uptake in NSC culture, isolated from rat SVZ and in in-vivo system. The EphA4 receptor-specific uptake was confirmed by performing immunocytochemistry and co-immunoprecipitation. After that, the cytotoxicity and cellular proliferation were assessed using MTT assay and BrdU assay, respectively. The rejuvenation

capability of Exo-pep-11 was assessed by checking the expression of markers like Nestin, ID1, PTEN, and AKT using immunocytochemistry and western blot. Finally, the neurogenesis potential of Exo-pep-11 was assessed by performing a western blot using protein isolated from an aging rat olfactory bulb. Together, our results demonstrate the dual potential of Exo-Pep-11 in NSC targeting, rejuvenation of NSC, and neurogenesis in aging adults. Our results indicate that Exo-Pep-11 has the potential to induce NSC proliferation subsequent neurogenesis and can find its application in Traumatic brain injury, Stroke, and neurodegenerative diseases.

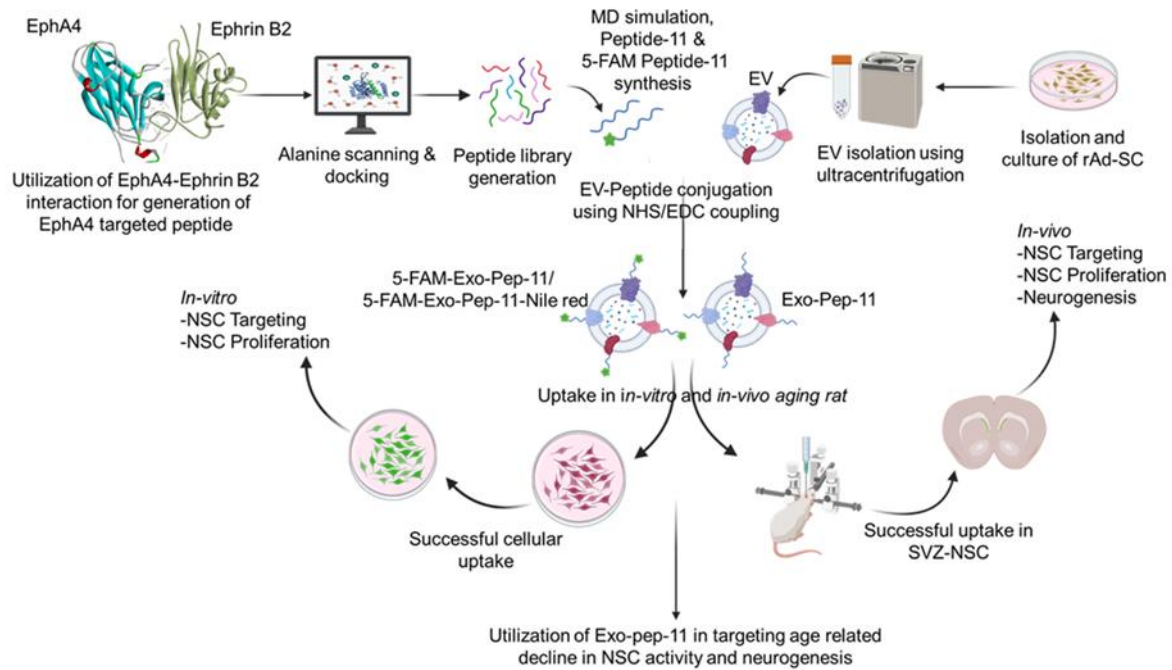


Figure 2.1. Schematic representation of the overall strategy of our work. The study includes computational designing and screening of EphA4 targeted peptide, surface functionalization of EV with Peptide 11, and investigation of the effectiveness of this engineered EV, Exo-pep-11, in targeting age-related decline in NSC activity & neurogenesis. MD: Molecular dynamics, EV: Extracellular vesicle, rAd-SC: rat adipose tissue stem cell, NSC: Neural stem cell, SVZ: Subventricular Zone. The picture was created using BioRender.

2.3 EXPERIMENTAL SECTION

2.3.1 Computational designing and identification of NSC targeting peptide

We utilized DrugscorePPI to conduct alanine scanning mutagenesis on the interface between EphA4 and ephrinB2 complexes (PPIs)⁴³. This technique involved systematically replacing amino acid residues with alanine to gauge changes in binding free energy ($\Delta\Delta G$) of PPIs. Our objective was to pinpoint the pivotal amino acids responsible for interactions at the interface. Initially, we uploaded the EphA4-ephrinB2 complex (2WO2.pdb) onto the server. Subsequently, mutagenesis was executed on the ephrinB2 chain. During this process, all hydrogens and non-peptide residues were automatically removed from the PDB file. The mutations were tracked, and the differences in binding free energy between the wild-type and mutated proteins were calculated. Based on these binding free energy values, residues exhibiting energy changes of ≥ 2 kcal/mol were categorized as hot spots, signifying their crucial role in the interactions at the interface area. Additionally, residues with energy changes ≥ 1 kcal/mol but ≤ 2 kcal/mol were identified as warm spots, indicating their significant involvement in the interactions as well. The residues with the highest energy changes are selected, along with some up and downstream residues. After that, the peptide library was

generated by overlapping sequence using the genscript peptide library tool (https://www.genscript.com/peptide_screening_tools.html). Then, some sequences were changed with counter amino acids to decrease the problem during peptide EV coupling.⁴⁴ The peptide sequences were built, and geometry was optimized using Avogadro 1.2.0 software. RCSB database was used to get the crystal structure of the target protein EphA4 (PDB: 2WO2). Then, the protein was prepared using BIOVIA Discovery Studio 2020 client by removing the existing ligand. PDBQT file of ligand and receptor was generated using AutoDockTool 1.5.7. Blind docking between EphA4 and Peptides was conducted (grid box size 46 x 50 x 62) using Autodock-Vina software version 1.1.2⁴⁵. After docking was completed, the binding affinity was analysed from the generated log file. Images and interactions were analysed and visualized using Pymol software. Peptides with binding affinity less than -7 kcal/mol are selected for further analysis. GROMACS 5.0.1 molecular dynamics package was used to simulate all the peptide-receptor complexes and only receptors in CHARMM 27 all-atom force field (CHARM 22 plus CMAP for protein) for 10 ns^{46,47}.

2.3.2 Synthesis of peptide 11, labeling peptide 11 with 5-FAM, and characterization

The peptide was synthesized using the microwave-assisted solid-phase peptide synthesis technique with a CEM-LIBERTY manual microwave peptide synthesizer, as discussed previously⁴⁸. Fmoc-Rink Amide was employed as the solid-phase support. In the deprotection step, a solution containing 20% v/v piperidine in DMF was used to eliminate the Fmoc protecting group. Diisopropylethylamine (DIPEA) (10 equivalents) and HBTU (5 equivalents) were utilized as the base and coupling agents, respectively. The peptides attached to the resin were liberated from the solid support through treatment with a cleavage mixture containing TFA, EDT, H₂O, and phenol at a volumetric ratio of 94:2.0:2.0:2.0 (v/v/v/v). The reaction mixture was stirred at room temperature for 4 hours. Excess TFA was eliminated by evaporating with a nitrogen gas flow until the volume was minimized. The residual solution was gradually introduced to 40 mL of ice-cold diethyl ether, leading to the formation of a curdy white precipitate. To guarantee thorough precipitation, the mixture was maintained at -20 °C overnight. The resulting precipitate was subsequently centrifuged, dissolved in a minimal volume of a 1:1 water and acetonitrile solution, and subjected to lyophilization for subsequent processing. For 5-carboxyfluorescein(5-FAM) peptide labeling, before peptide cleavage from the resin, the peptide was reacted with 5-carboxyfluorescein (3 equiv.) in the presence of DIC (3 equiv.) and HOBt (3 equiv.) for 24 hr in room temperature under gentle shaking. Then, the resin-bound 5-FAM tagged peptide is washed 3 times, and the peptide is cleaved as previously described. Peptide 11 and 5-FAM peptide 11 were characterized by ESI-MS mass spectrometry in positive mode (Agilent 6530). A Shimadzu LC-20AP HPLC system was utilized to analyze the purity of the peptide 11 and 5-FAM peptide 11 in a C18 column. Acetonitrile in water, in the form of a linear gradient (0-20 min, 50-70% acetonitrile), was used to elute the molecules at a flow rate of 1 mL/min, and the absorbance of the effluent was measured at 220 nm.

2.3.3 Isolation and characterization of rAd-SC from adult rat

All animal studies were conducted with approval from the Institutional Animal Ethics Committee (IAEC) of JNVU, Jodhpur, India, following the guidelines outlined by the Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The Isolation was done following the previously described protocol⁴⁹. Briefly, the fat tissue surrounding the epididymis was isolated from 2- to 3-month-old male rat epididymal fat pads, which were meticulously cut and placed in a Petri dish containing phosphate-buffered saline (PBS). Using forceps and a scalpel, the fat pads were cut into small pieces and transferred to a 50 mL tube containing 10 mL of sterile PBS. The minced fat tissues

underwent five washes with 10 mL of PBS each time to eliminate contaminating blood. After washing, the adipose tissue was suspended in 10 mL of collagenase solution (0.1% collagenase type 1 in PBS). The tube was tightly sealed with parafilm and incubated in a 37 °C incubator for 45 minutes with occasional mixing until the tissue achieved homogeneity. Upon completion of collagenase digestion, the tube was vortexed for 15 seconds, followed by centrifugation for 5 minutes at 300 x g. The tube was vortexed again for 10 seconds and then underwent another 5 minutes of centrifugation, resulting in the formation of three layers. The oil layer was carefully discarded, followed by the aqueous layer, without disturbing the stromal vascular fraction (SVF) pellet. The SVF pellet was resuspended in 10 mL of sterile BSA solution (1% albumin, bovine serum Fraction V solution in PBS). After 5-minute centrifugation at 300 x g, the supernatant was discarded, and the SVF pellet was suspended in 8.5 mL of complete media containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 2 mM L-glutamine. The cells were cultured in a 25 cm² flask at 37 °C under 5% CO₂. The next day, attached cells were examined, suspended cells were removed, and the media was replaced. Subsequently, the media was changed every other day. The cells were passaged every 5-7 days when they reached 80%-90% confluence using Trypsin-EDTA. The isolated cells were characterized by the presence of CD29 (1:500, Armenian hamster monoclonal, MA5-16708, Invitrogen) and CD 44 (1: 1000, Rabbit monoclonal, ab189524, Abcam) and the absence of CD31 (1:1000, mouse monoclonal, CBL1337, Millipore) using immunocytochemistry.

2.3.4 EV isolation from rAd-SC culture supernatant

The EV isolation was done following the previously discussed protocol ⁵⁰. Briefly, 10⁶ rAd-SCs were seeded in each T75 flask in media containing DMEM/F12 and 10% FBS media and kept at a 37°C incubator. When the culture reached 70% confluency, the flasks were washed twice with PBS and cultured for 48 h in EV-free expansion medium (DMEM/F12 + 10% EV-depleted FBS). The number of viable cells was assessed, and media supernatant was collected. The supernatant was then differentially centrifuged at 300 x g for 10 min, then 2000 x g for 10 min, 10,000 x g for 30 min, 100,000 x g for 70 min, and finally 100,000 x g for 70 min. The pellet from the final centrifugation contains the EV. The pellet was then dissolved in 100 µL PBS-HAT (1X PBS with albumin 0.2 %; Trehalose 25 mM; HEPES 25 mM) and stored at - 80 °C ⁵¹.

2.3.5 Functionalization of EV with peptide 11 (Exo-pep-11) and 5-FAM peptide 11 (5-FAM-Exo-pep-11)

After peptide synthesis was done, the EV was functionalized with the peptide using acid-amine coupling. The Carboxy group present in the peptide will be coupled to the amine group present in the protein on the surface of EV using EDC/sulfo-NHS in MES buffer. 25 µL of 10 mg/mL peptide 11 was added to 25 µL of 20 mg/mL EDC, and 25 µL of 20 mg/mL of sulfo-NHS solution in 100 mM, pH 6 MES buffer, and the carboxyl group was activated on a shaker for 30 min. Then 100 µL of 1 mg/mL EV was added to the mixture, and the pH of the reaction mixture was increased to above 7 by adding concentrated PBS. The reaction mixture is incubated on a shaker for 3 hr. At the end of the reaction amide bond will form between EV and peptide. After the reaction, the excess unreacted compound was washed using a 100k cutoff amicon filter, applying 1 x PBS. The same procedure was repeated twice to ensure maximum coupling of peptide with EV.

2.3.6 Characterization of EV and Exo-pep-11

For TEM analysis, the EVs were fixed with Karnovsky's fixative for 1 hr at 4 °C. Then the EVs were washed with phosphate buffer and applied to the E.M. grid. After that, the EVs were negatively stained with uranyl acetate and finally visualized using HR-TEM (Tecnai).

For FTIR analysis, a drop EV sample was applied and analyzed in ATR mode. Scanning was done from 500 to 4000 cm⁻¹. In all, FTIR run solvent was kept as a blank sample to normalize the peaks.

The size or diameter of the EVs was determined using dynamic light scattering (DLS). A Gaussian function was applied to the measured size distribution to calculate the mean hydrodynamic diameter of the EVs. To dissolve potential EV aggregates, the EV samples were shaken at 4 °C for 20 minutes. Approximately 50 µL of each sample was placed in a cuvette with a 10 mm path length. DLS measurements were carried out at 20 °C using a Nano Zetasizer (Malvern Instruments Ltd., U.K.).

For western blot analysis, the EVs were lysed in RIPA buffer containing 1% protease and phosphatase inhibitors. The protein was quantified using Bradford assay. Then, the lysate was dissolved in SDS-PAGE sample buffer, boiled for 5 min at 95 °C, subjected to 12% SDS-PAGE, and then blotted onto PVDF membranes (Merck). Membranes were blocked for 1.5 h at room temperature with 5% BSA in TBST and then incubated overnight at 4 °C with the following primary antibodies: mouse anti-CD81 (1:500, MA5 13548, Invitrogen), mouse anti-tsg101 (1:1000, sc-7964, Santa Cruz), rabbit anti- α tubulin (1:5000, ab52866, Abcam) and mouse anti-Col1A1 (1:1000, sc-293182, Santa Cruz). After 3 times wash with TBST, membranes were incubated for 2 h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies and imaged using G-box chemi XRQ apparatus (Syngene, UK).

2.3.7 Isolation and characterization of adult rat SVZ-derived NSC

The Isolation of NSC was done following the previously discussed protocol⁵². The brains were extracted from 12- to 13-month-old Wister rats, and the SVZ, a thin tissue layer surrounding the ventricle, was meticulously dissected. This involved placing one blade of fine curved forceps in the lateral corner of the lateral ventricle just under the corpus callosum and the other approximately 1 mm into the tissue adjacent to the ventricle. The dissected SVZ tissues were then placed in a Petri dish on ice. Using a scalpel blade, the tissues were minced for about 1 minute until no large pieces remained and transferred to a 15 mL tube with 1 mL of pre-warmed 0.05% Trypsin-EDTA. The tissues were incubated for 7 minutes in a 37 °C incubator. The enzymatic reaction was halted by adding 1 mL of trypsin inhibitor containing DNaseI, and the contents were mixed by flicking the tube. The solution was centrifuged at 300 x g for 5 minutes, and the pellet was resuspended in 1 mL of growth medium. The tissue was dissociated by gently pipetting up and down approximately 7-10 times using a P1000 pipette. Subsequently, the dissociated tissue was centrifuged at 300 x g for 5 minutes, and the pellet was dissolved in 200 µL of NSC medium containing Neural Basal Medium with 2% B27, 1x GlutaMAX, 2 µg/mL heparin, 50 units/mL Penicillin/Streptomycin, 20 ng/mL purified mouse receptor-grade epidermal growth factor (EGF), and 20 ng/mL recombinant bovine fibroblast growth factor (FGF-2). Finally, the cell fraction was plated in a confocal dish coated with poly-L-ornithine (50 µg/mL) and laminin (5 µg/mL). After 24 hours, the culture media were replaced with fresh media, and subsequently, half of the media was replaced every 48 hours until the cells reached 70-80% confluence. For subculturing, the cells were detached from the dish surface by adding Accutase. To generate neurospheres, the Accutase-dissociated cells were resuspended in media and plated in an uncoated confocal dish or 12-well plate as needed. The isolated cells were characterized using immunocytochemistry with

Nestin (1:500, ab6142, Abcam) and ID1 (1:300, sc133104, Santa Cruz), which are specific markers for neural stem cells.

2.3.8 Cellular uptake and co-localization analysis of 5-FAM-Exo-Pep-11 and 5-FAM-Exo-Pep-11-Nile red

A total of ~50 neurospheres were seeded in a 35 mm confocal dish pre-coated with poly-L-ornithine (50 µg/mL) and laminin (5 µg/mL). After 24 hr of culture, cells were treated with 5-FAM-Exo-Pep-11 or 5-FAM-Exo-Pep-11-nile red, incubated for 30 min, and subsequently, cells were fixed using 4% Paraformaldehyde (PFA). The fixed cells were immune-stained with Nestin (1:500, ab6142, Abcam) and EphA4 (1:300, sc365503, Santa Cruz), and images were captured using Olympus IX 83 fluorescent microscope with appropriate fluorophore tagged secondary antibody.

2.3.9 Cytotoxicity analysis of peptide 11 and Exo-pep-11 in neural stem cell culture

10⁴ NSC were seeded per well in 96 well plates and cultured in NSC culture media for 24 hr. After that, the cells are treated with 400, 200, 100, 50, 25, 10, 5, and 1 µM and 1, 5, 10, 20, 40, 80, and 100 µg/mL concentrations of peptide 11 and Exo-pep-11 respectively. The cells were incubated for another 48 hr. Then MTT (5 mg/mL) was added in all the wells except the blank well and kept for 4 hr. Finally, the insoluble formazan was dissolved in 1:1 DMSO and methanol, and the absorbance was taken through the microplate reader at 570 nm.

2.3.10 BrdU cell proliferation assay

The assay utilized the BrdU Cell Proliferation Assay kit (2750, Millipore). Each well of a 96-well plate was seeded with 10⁴ NSC and incubated for 24 hours at 37 °C. Subsequently, Exo-pep-11 (40 µg/mL) and Unmodified EV (40 µg/mL) treatment was administered and maintained for up to 72 hours. BrdU was introduced into the cultured wells 12 hours before the conclusion of the exo-pep-11 incubation. Following incubation, the media were removed from the cell wells, and 200 µL/well of Fixing Solution was added, incubating at room temperature for 30 minutes. The fixing solution was then aspirated, and the plate was allowed to air dry. The plate was washed three times with 1X Wash Buffer. After the final wash, excess liquid was removed by blotting the plate on paper towels. A 100 µL/well of Anti-BrdU monoclonal antibody was added and incubated for 1 hour at room temperature. The wells were washed three times with wash buffer and then incubated with 100 µL/well of Goat anti-Mouse IgG secondary antibody for 30 minutes at room temperature. The plate was washed again three times with wash buffer, and 100 µL/well of TMB Peroxidase Substrate was added, incubating for 30 minutes at room temperature in the dark. Finally, 100 µL/well of the acid stop solution was added, and the absorbance was measured using a microplate reader set at dual wavelengths of 450/550 nm.

2.3.11 Expression analysis of Nestin and ID1 in NSC culture

~50 neurospheres were seeded in poly-L-ornithine /laminin-coated 35 mm confocal dish and incubated for 24 hr at 37 °C incubator. The cells were then treated with 40 µg Exo-pep-11 or Unmodified EV and incubated for 48 hrs. The cells were then fixed in 4% PFA, permeabilized, and incubated with primary antibodies of Nestin (1:500, ab6142, Abcam) and ID1 (1:300, sc133104, Santa Cruz), overnight. Then, the cells were washed and incubated with anti-mouse 594 secondary antibodies (1:500, ab150116, Abcam) and imaged using a fluorescence microscope (Olympus, IX83).

2.3.12 Calculation of peptide modification efficiency

The quantity of protein derived from EV before and after peptide coupling was verified using the Bradford Protein Quantitation assay. Specifically, Bradford Protein Quantitation was performed after gradient dilution of a known concentration of EVs, and the standard curve was depicted. Following that, the EV concentration and quantity were determined using the standard curve. The calculation of modified polypeptides on EVs involved the use of the following equation. In this equation, “M” represents the molecular weight of polypeptides, and “m” denotes the mass of polypeptides conjugated on EVs, derived from the difference between the EV mass and the total protein content.

$$N(pep/ml) = \frac{m}{M} \times N_A$$

2.3.13 EV staining with Nile red

EVs were subjected to incubation with Nile Red at a final concentration of 10 µg/mL at 37 °C for 30 minutes. Subsequently, the samples underwent washing with PBS and were resuspended in PBS. Fluorescence emission was observed by exciting the solution excited with 550 nm laser.

2.3.14 EV staining with Dil

EVs were incubated with Dil at a final concentration of 2 µM in PBS at 37°C for 20 minutes. Following this incubation, the excess dye was removed using a cutoff filter, and the labeled EVs were washed twice with PBS. The resulting labeled EVs were then utilized for a cellular uptake study in NSC cultures.

2.3.15 Determination of binding affinity using tryptophan fluorescence quenching

EphA4 was isolated from total rat brain lysate using immunoprecipitation via EphA4 antibody coupled agarose bead. To evaluate the binding affinity of Peptide 11 for EphA4, a tryptophan fluorescence quenching assay was employed. Solutions containing varying concentrations of EphA4 (ranging from 2 to 40 µM) and a fixed concentration of Peptide 11 (2 µM) were prepared in 20 mM Tris buffer, pH 8. These solutions were incubated at 4°C for 40 minutes. Fluorescence measurements were then taken with excitation at 280 nm, and emission was detected between 310 nm and 400 nm. The resulting data were analyzed using a modified Stern-Volmer equation and further processed with GraphPad Prism 8.0 for binding affinity calculation.

2.3.16 Stereotactic injection and uptake analysis in *in vivo* system

Male adult Wistar rats (12-13 months old, n=6) were housed under controlled standard conditions in separate cages, with a temperature of 25 ± 2 °C and a relative humidity of 50%. For surgical procedures, the rats were anesthetized via intraperitoneal injection with a blend of ketamine 10% (80 mg/kg) and xylazine 2% (10 mg/kg). The rats were then secured onto a stereotaxic apparatus (Stoelting), and a sagittal incision was made in the skin to expose the skull. A small hole was drilled into the cranium at specific coordinates (-0.8 mm anterior and 1.6- or 2 mm lateral to the bregma) for injecting into the right lateral ventricle or the subventricular zone, respectively. Using a 10-µL Hamilton syringe, a 20-µL volume of 100 µg exo-pep-11 or 5-FAM-Exo-Pep-11 or 5-FAM-Exo-Pep-11-nile red was injected at a rate of 0.5 µL/minute, at a depth of 4.3 mm from the outer border of the cranium. The incision in the head skin was then sutured. The rats were euthanized 24 hours after the injection, and their

brains were removed and rapidly frozen using isopentane and liquid nitrogen. The frozen brains were stored at -80 °C before being sectioned using a cryostat (Cryotome FSE, Thermo). For immunohistochemistry, brain sections (10 µm thick) were fixed in cold methanol for 10 minutes, washed three times in PBS, and blocked with PBS containing 5% bovine serum albumin at room temperature for 1 hour. When required, the fixed tissues were then immunostained with Nestin (1:500, ab6142, Abcam) and EphA4 (1:300, sc365503, Santa Cruz) and examined using an Olympus IX 83 fluorescent microscope with appropriate fluorophore-tagged secondary antibodies.

2.3.17 Western blot analysis of rat brain tissue lysate

Male Wistar rats (12-13 months old) were separated into two groups, each containing ten rats. The treatment group rats were anesthetized via intraperitoneal injection with a combination of ketamine 10% (80 mg/kg) and xylazine 2% (10 mg/kg), and EVs (40 µg and 100 µg of Exo-pep-11 or 40 µg and 100 µg of Unmodified EVs) were stereotaxically injected into the lateral ventricle. Rats were euthanized after 3 to 7 days, and their brains were carefully removed. Specific brain regions for protein isolation were dissected and homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors. Following protein quantification using the Bradford method, samples were subjected to 10-12% SDS-PAGE and then transferred onto PVDF membranes. The blots were blocked with 5% BSA in TBST and incubated with primary antibodies, including Nestin (1:500, ab6142, Abcam), ID1 (1:300, sc133104, Santa Cruz), Phospho-Akt (Ser473) (1:500, 05-1003, Merck Millipore), Phospho-PTEN (Ser380) (1:1000, 9551T, CST), TUJ1 (1:1000, MAB1637, Invitrogen), and TH (1:500, MA1-24654, Invitrogen) overnight at 4 °C. Subsequently, the blots were incubated with the appropriate HRP-conjugated secondary antibodies and visualized using the G-box chemi XRQ apparatus (Syngene, UK).

2.3.18 Confirmation of receptor-specific uptake of Exo-pep-11

To check whether NSC takes up the Exo-pep-11 in a receptor-targeted manner, we have performed two experiments: one is cellular uptake after antibody treatment, and the other is a coimmunoprecipitation assay. For the first experiment, 10⁴ NSCs were seeded in a laminin/ornithine coated 35 mm dish; after 24 hr incubation, when the cells got adhered, the cells were treated with anti EphA4 primary antibody for 1 hr. in 37 °C. Then, the cells were treated with 5-FAM-Exo-pep-11 and incubated in a 37 °C incubator for 4 hr. Finally, the cells were fixed with 4 % paraformaldehyde, and images were captured using a fluorescent microscope (IX 83, Olympus). For the co-immunoprecipitation experiment, the protein was isolated from rat brain SVZ and incubated with anti-EphA4 primary antibody and Pep-11 in two different combinations. In one combination, the protein was incubated with Pep-11 and then with primary antibody, and in the second combination, the protein was first incubated with primary antibody and then with Pep-11. After that, the Agarose A/G bead was added and incubated for 4 hr in 4 °C. Finally, the mixture was centrifuged at 2500 rpm for 5 min in 4 °C. The supernatant and pellet were then used for dot blot and mass spectroscopic analysis to check the presence of a protein of interest and Pep-11, respectively.

2.3.19 Data analysis and statistics

The analysis of microscopic images was conducted using ImageJ software. Each data point in the results represents the mean of a minimum of three replicates, with error bars indicating $\pm$ SD. For graph plotting and statistical analysis, we used GraphPad Prism 8.0.2 software. Statistical analyses, where applicable, involved one-way ANOVA followed by Tukey's post-test for comparison or Student's t-test. Significance levels were denoted as follows: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001, and ns for not significant. Some components of visual illustration were made using BioRender.

2.4 RESULTS AND DISCUSSION

2.4.1 Peptide library designing, screening, EV surface functionalization, and characterization of EV and Exo-pep-11

Alanine scanning was performed to find out the important residues in the EphA4-Ephrin B2 interaction (Figure 2.5 A, B). The residues were then used to generate a peptide library (Figure 2.4 A), and docking was performed with the EphA4 receptor to calculate the binding affinity (Figure 2.5 C). The peptide with a binding affinity of less than -7 kcal/mol was selected for further computational analysis. Peptides 1, 6, 7, and 11 were used for simulation analysis for 10 ns. From the RMSD plot (Figure 2.4 B), we have selected peptide 11, as it fluctuates at lower RMSD values as compared to other peptide-protein complex, which indicates higher structural stability. We also conducted cytotoxicity assessments on NSC cultures using peptides 1, 6, 7, and 11, revealing that Peptide 11 exhibited the highest cytocompatibility compared to the other three peptides (Figure 2.14 A and Figure 2.3). Following computational screening and cytotoxicity analysis, we identified peptide 11 as a promising candidate for targeting NSCs. Subsequently, we synthesized peptides 11 and 5(6)-carboxyfluorescein coupled peptide 11 (5-FAM-pep-11) and used them for surface functionalization of the EV isolated from rat adipose tissue stem cells (Figure 2.2).

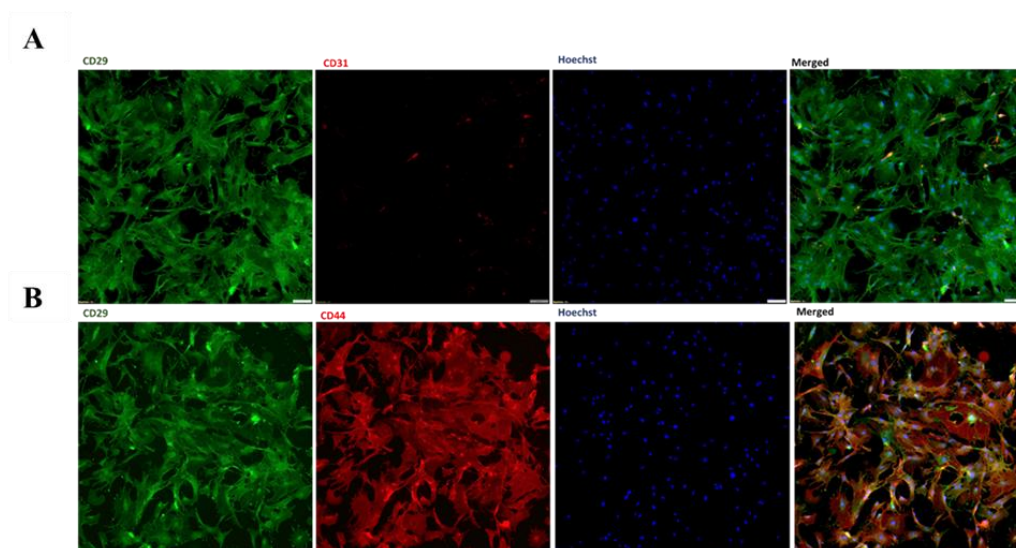


Figure 2.2 Characterization of Rat Ad-MSC (A) CD29 and CD31 expression in isolated cells. (B) CD29 and CD44 expression in isolated cells. Scale bar represents 100 μ m.

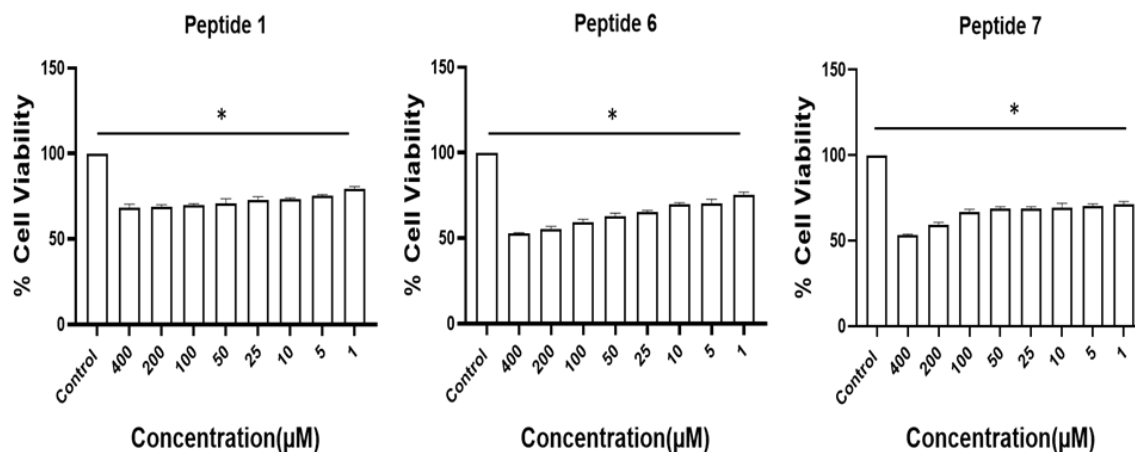


Figure 2.3 Cytotoxicity assay data plot for peptide 1,6,7.

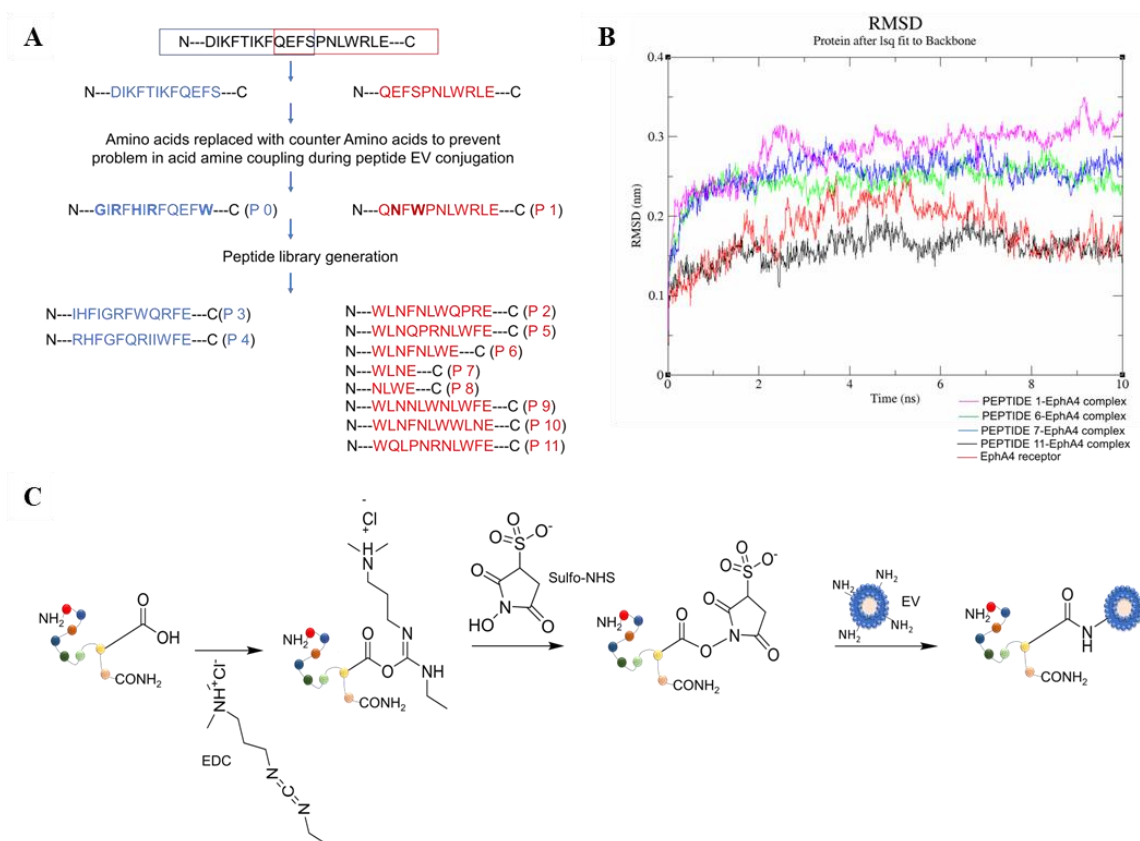


Figure 2.4 Computational peptide designing and EV functionalization strategy. (A) Construction of peptide library using computational analysis. Amino acids in bold represent counter amino acids (P0 and P1). (B) RMSD plot of EphA4 receptor with selected peptide and EphA4 free receptor simulation for 10 ns, performed in GROMACS. (C) Schematic representation of functionalization strategy of EV with designed peptides.

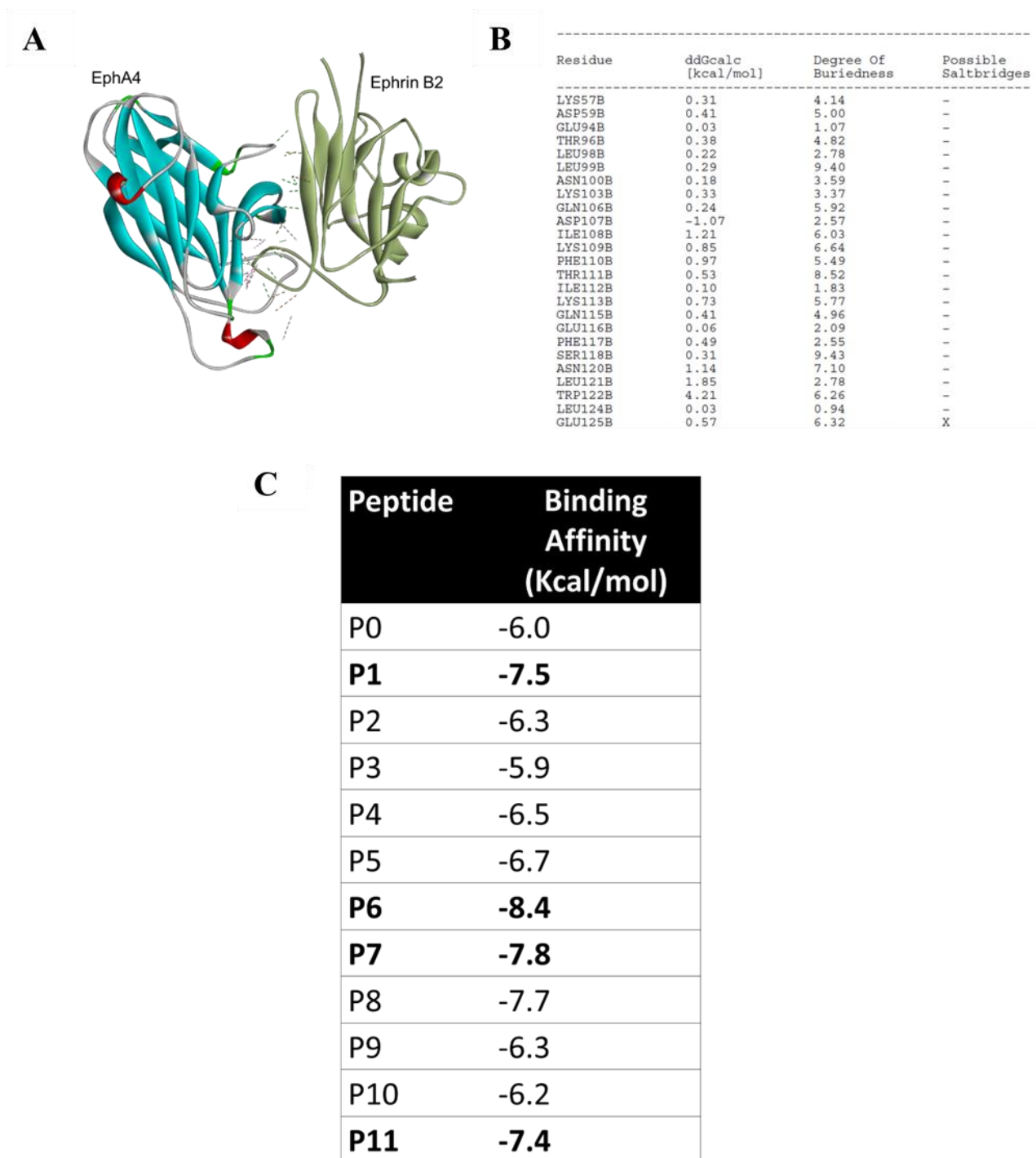


Figure 2.5 Peptide designing strategy. (A) Representation of EphA4-Ephrin B2 interaction. (B) Alanine scanning data shows the important residue for the EphA4-Ephrin B2 binding. (C) Docking data of designed peptide library with EphA4 receptor. P: Represents peptide.

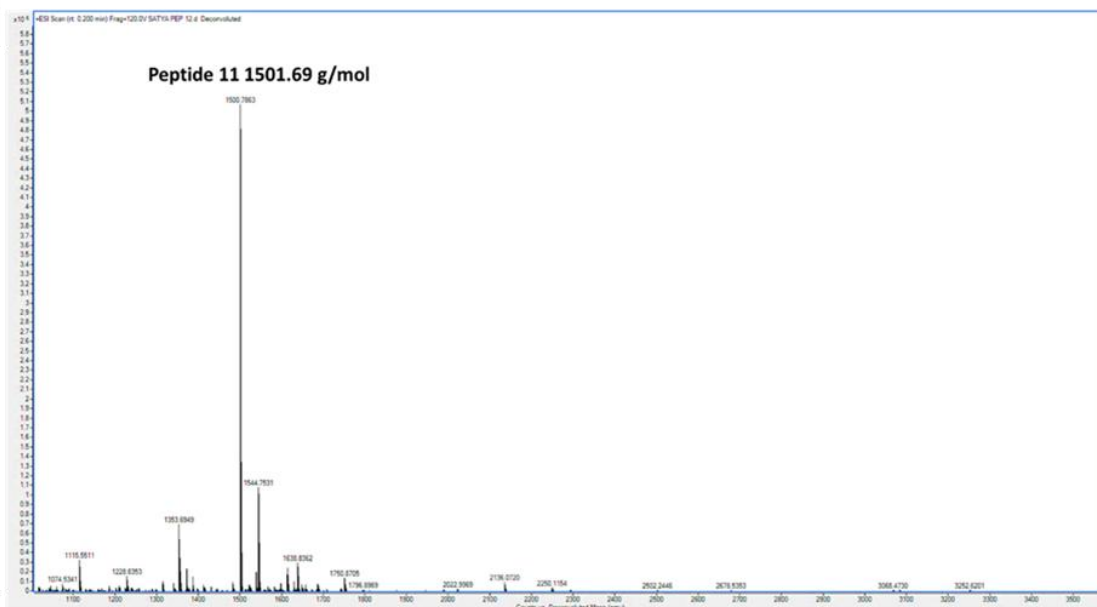
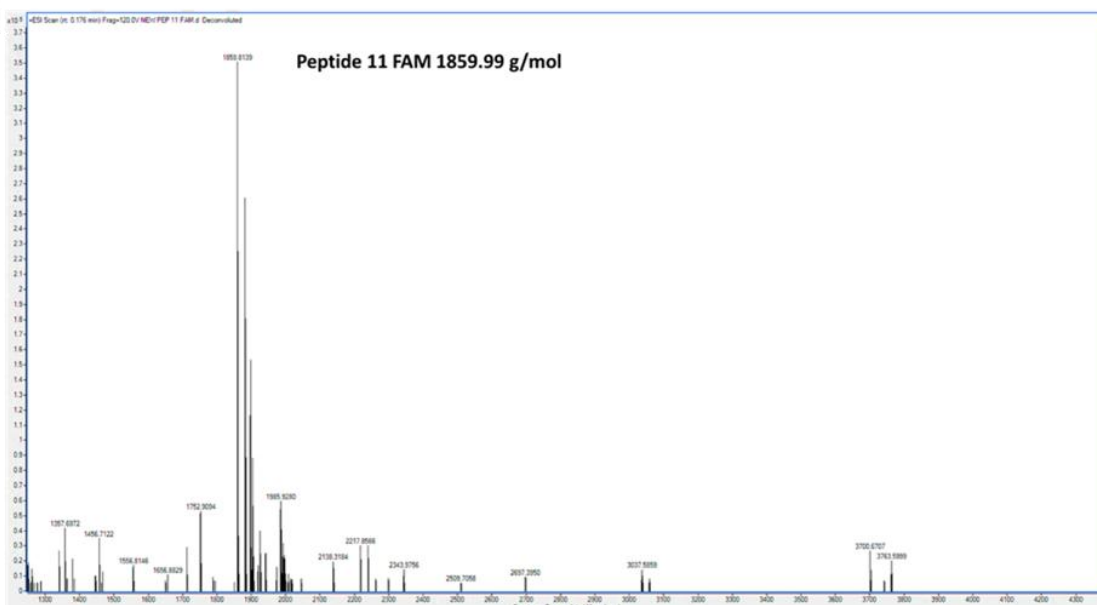
A**B**

Figure 2.6 (A) Mass spectroscopy of Peptide 11. (B) Mass spectroscopy of Peptide 11 conjugated with 5-Carboxyfluorescein (5-FAM-PEP-11).

We have characterized and purified peptide-11 and 5-FAM-pep-11 using ESI-MS (Figure 2.6) and HPLC (Figure 2.7), respectively. The functionalization was performed between the carboxyl group present in the glutamic acid of peptide and the amine group of EV protein using sulfo-NHS/EDC acid amine coupling (Figure 2.4 C). After this step, we successfully developed a peptide-functionalized EV, Exo-Pep-11, which was used for further analysis. The engineered EV was characterized using TEM imaging (Figure 2.10 A, B), FTIR (Figure 2.10 C), and DLS (Figure 2.10 D, E) techniques. FTIR data shows a characteristic peak at $\sim 1637\text{ cm}^{-1}$, which is attributed to the stretching of carbonyl groups on $-\text{CONH}$. This phenomenon can be explained by the presence of enriched proteins on the EV membrane. Notably, the absence of absorption bands related to carboxyl stretching vibration at $\sim 1710\text{ cm}^{-1}$ following the amide reaction signifies the successful covalent linking of the carboxyl group of peptides to the amino groups on EV proteins.³⁸ DLS data confirms peptide conjugation as there is an increase in average particle size from $177.3 \pm 14.89\text{ nm}$ to $195.76 \pm 8.37\text{ nm}$. Further, the purity of EV

was confirmed by the presence of EV-specific marker CD 81 and tsg101 and less or absence of cell-specific marker β actin and Col1A1 by western blotting (Figure 2.10 F). We have also calculated the peptide modification efficiency. Under the optimum reaction condition, we calculated that approximately 3975 ± 40 Peptide 11 was conjugated onto one EV surface (Figure 2.8). To check the EV-peptide conjugation, we functionalized EV with 5-FAM labeled peptide 11 and stained the EV with Nile red. Nile red will only show fluorescence if it is bound to EV, and Nile red fluorescence increases with an increased concentration of EV (Figure 2.9 C). After a successful reaction and washing, the fluorescence emission of fluorophore-tagged peptide conjugated, Nile red stained EV (5-FAM-Exo-pep-11-Nile red) was recorded using a plate reader. Exo-pep-11 shows emission spectra corresponding to both 5-FAM and Nile red, confirming successful conjugation (Figure 2.9 A, B). All the above-discussed results confirm the purity of EV, successful synthesis of peptide 11, and successful peptide-EV covalent conjugation.

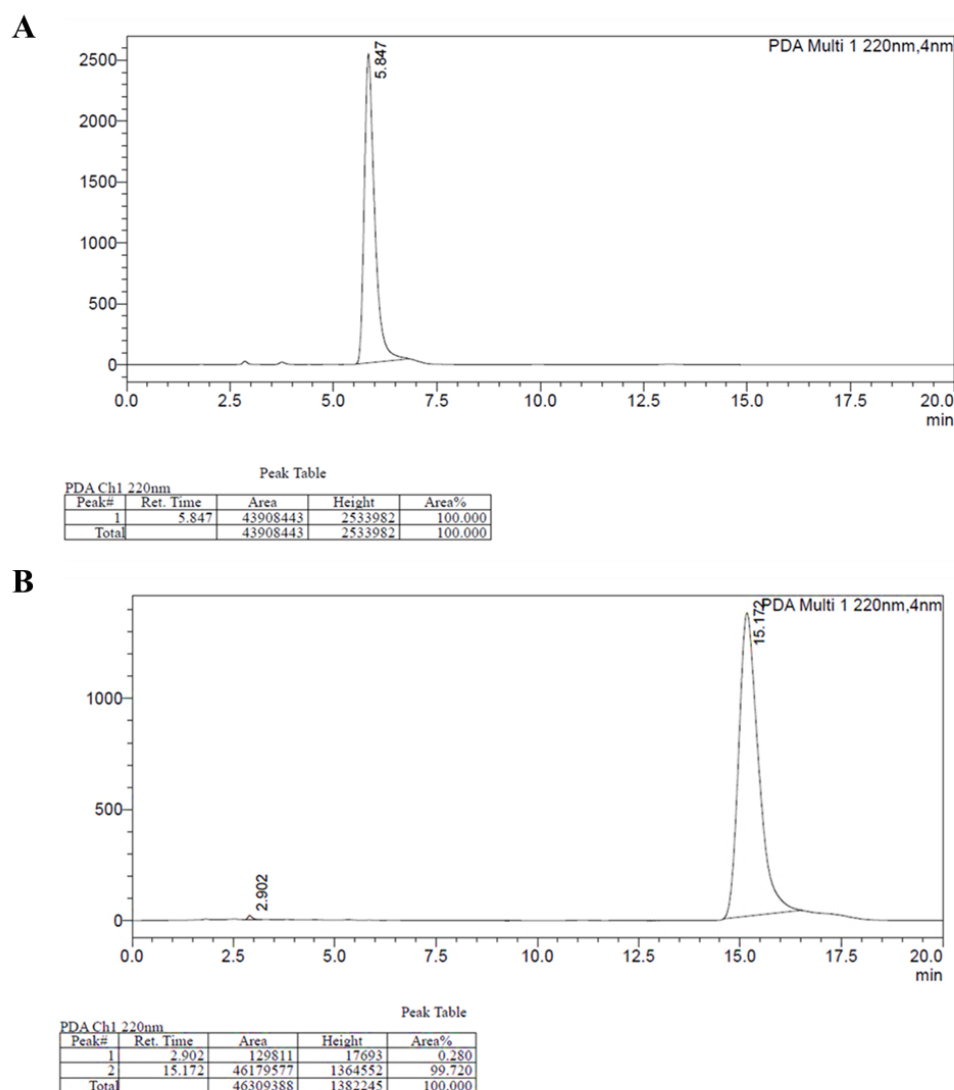


Figure 2.7 (A) HPLC chromatogram of Peptide -11 (B) HPLC chromatogram of 5-FAM-pep-11.

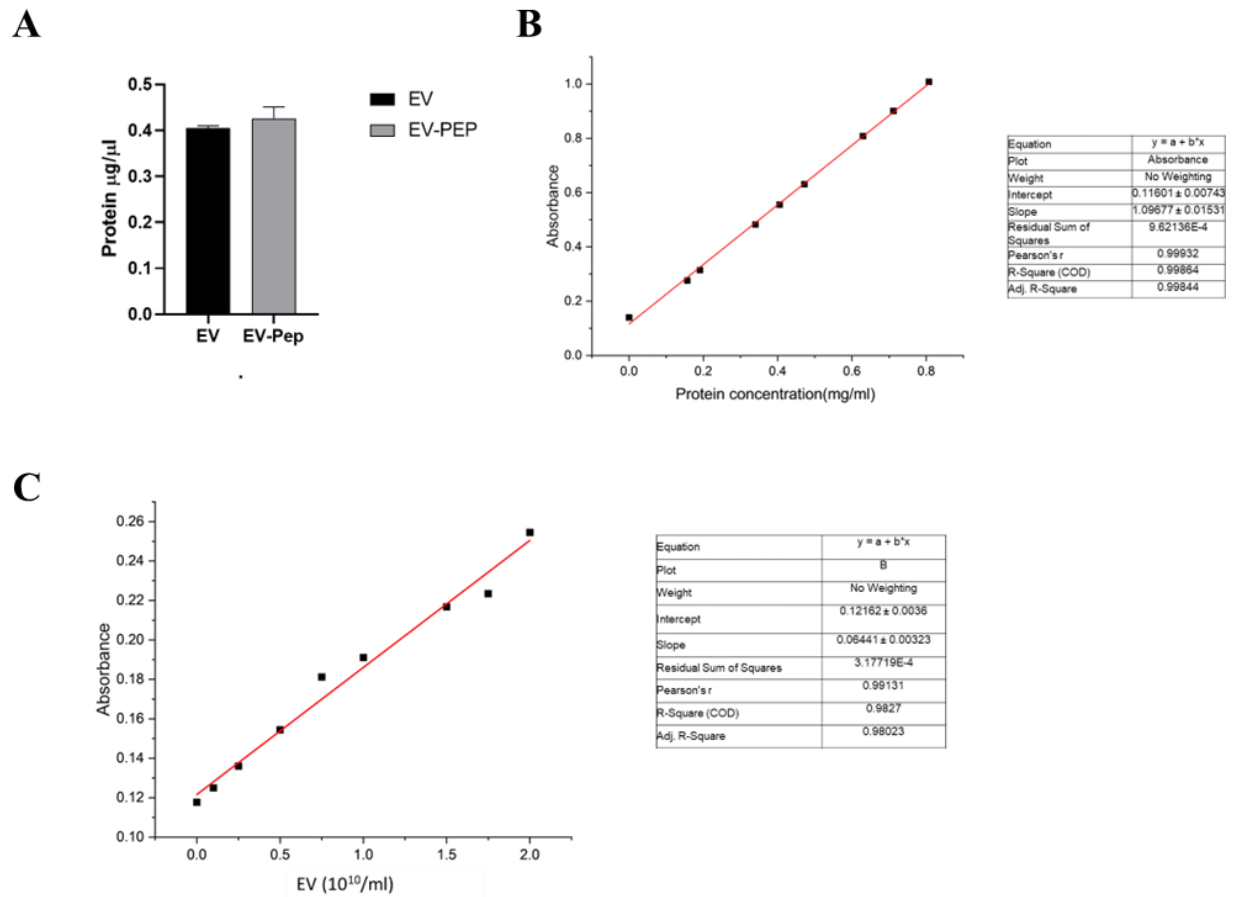


Figure 2.8 Calculation of peptide modification efficiency. (A) Quantification of protein after and before EV-peptide conjugation reaction. (B) Standard curve of absorbance versus the quantity of protein derived from EVs. (C) Standard curve of absorbance versus the concentration of EVs.

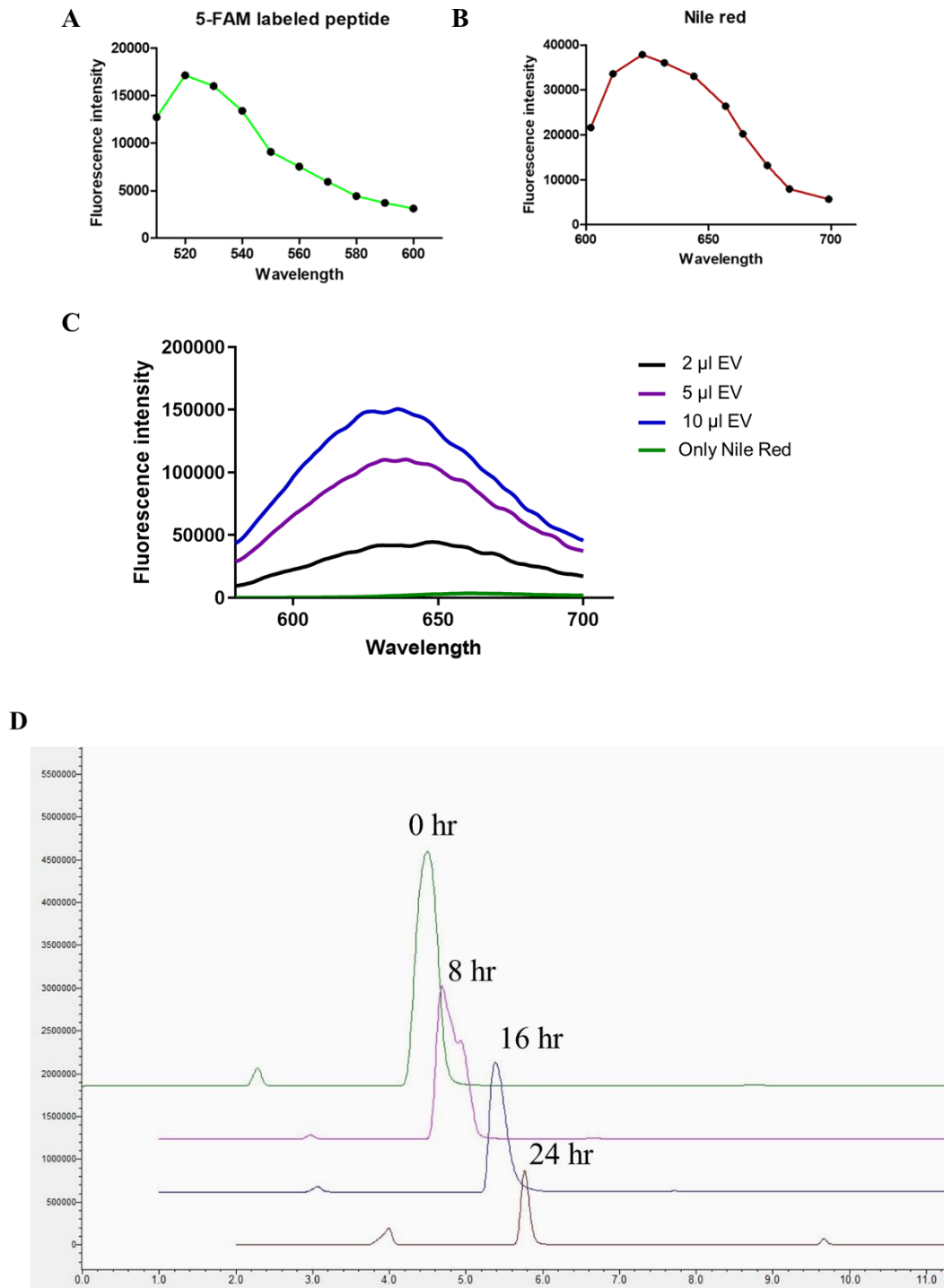


Figure 2.9 Fluorimetry analysis (A) 5-FAM-Exo-Pep-11-Nile red fluorescence emission spectra when excited with 490 nm lesser. The spectrum corresponds to 5-FAM. (B) 5-FAM-Exo-Pep-11-Nile red fluorescence emission spectra when excited with 550 nm lesser. The spectrum corresponds to Nile red. (C) Nile red fluorescence emission spectra in the presence of different concentrations of EV and absence of EV. (D) Serum stability analysis of Exo-pep-11.

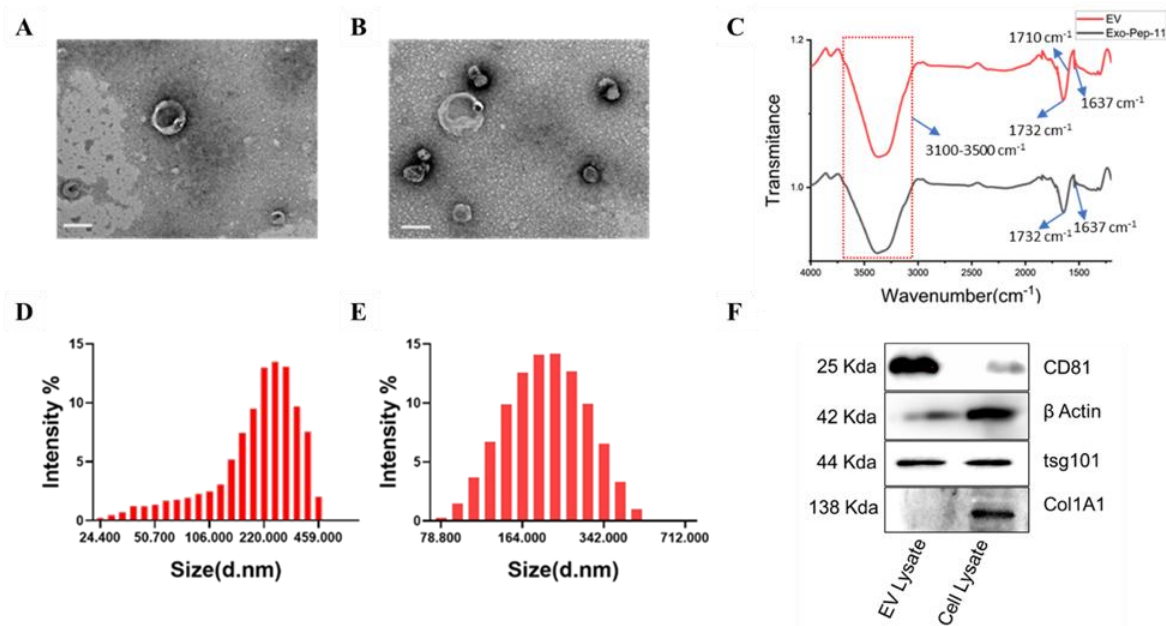


Figure 2.10 Characterization of rat adipose tissue stem cell-derived EV and Exo-pep-11. Transmission Electron Microscopy (TEM) images of (A) EV and (B) peptide-functionalized EV (Exo-pep-11). The scale bar represents 100 nm. (C) FTIR spectra of EV and Exo-pep-11. DLS data showing the size distribution of (D) EV and (E) Exo-pep-11. (F) Western blot analysis of EV marker CD81, tsg101, β Actin, and Col1A1.

2.4.2 Exo-pep-11 internalizes specifically by NSC and colocalizes with NSC-specific markers

To check the NSC targetability and further cellular effect, we isolated NSC from adult rat SVZ and cultured it as a freely floating neurosphere, adherent culture, or monolayer culture (Figure 2.11 A). Then, the cells were characterized by the presence of NSC-specific markers ID1 (Figure 2.11 B). To check the NSC targeting ability, the adherent NSC culture was treated with either 5-FAM-Exo-Pep-11 or 5-FAM-Exo-Pep-11-Nile red, and uptake was analysed by fluorescent microscopy (Olympus IX 83). Cells were further immune-stained with EphA4 and Nestin to check the colocalization of engineered EV with an NSC marker. The 5-FAM-Exo-Pep-11-Nile red-treated cells show a fluorescence signal in green as well as red channel, which indicates the conjugation of EV-peptide was successful and is successfully internalized by the cell (Figure 2.13 A). Immunocytochemistry staining confirms the uptake of Exo-pep-11 specifically to NSC as it is colocalized with NSC marker EphA4 (Figure 2.13 B) and Nestin (Figure 2.13 C). Images were also analyzed using the ImageJ plugin colocalization finder to confirm the colocalization of Exo-Pep-11 with NSC markers (Figure 2.12). To check that peptide conjugation has any effect on cellular uptake, we have labeled the bare EV and Exo-pep-11 with Dil, and the results demonstrated that Exo-pep-11 has ~ 1.62-fold higher cell uptake as compared to the bare EV (Figure 2.15).

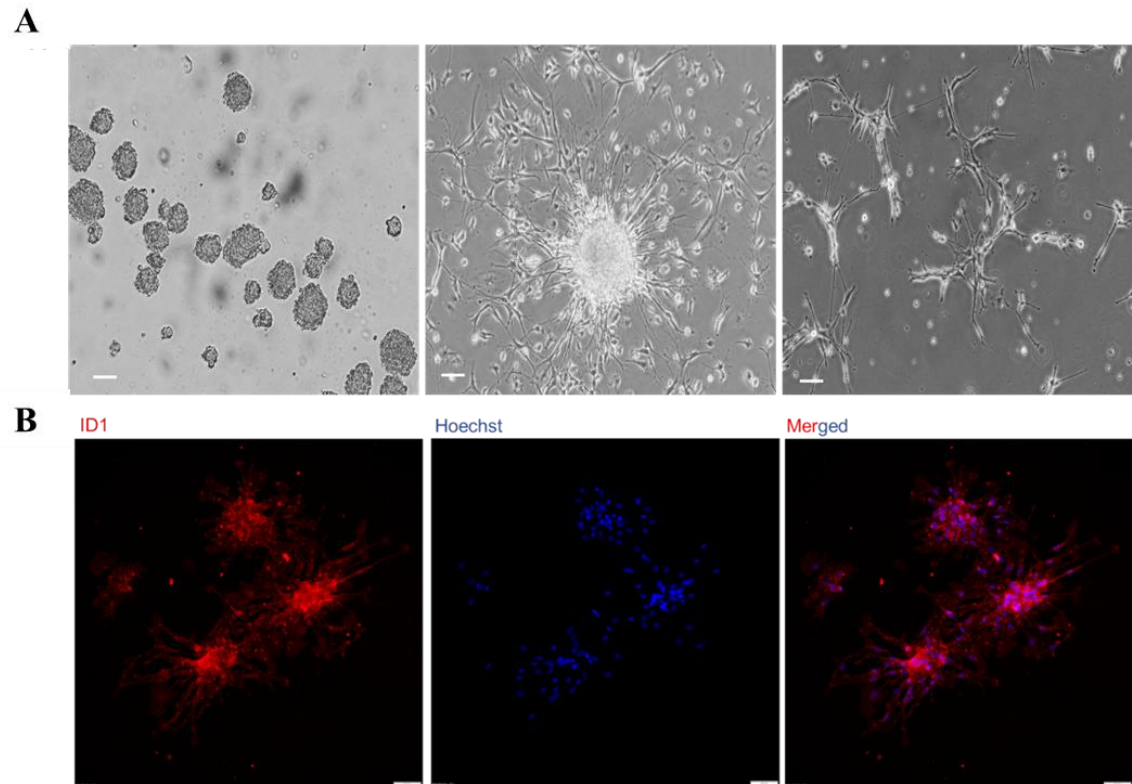


Figure 2.11 Characterization of adult rat SVZ-derived NSC. (A) Representative DIC images of NSC cultured as freely floating neurosphere, adherent culture, and single-cell adherent culture. (B) characterization of NSC by ID1 expression. The scale bar represents 50 μ m.

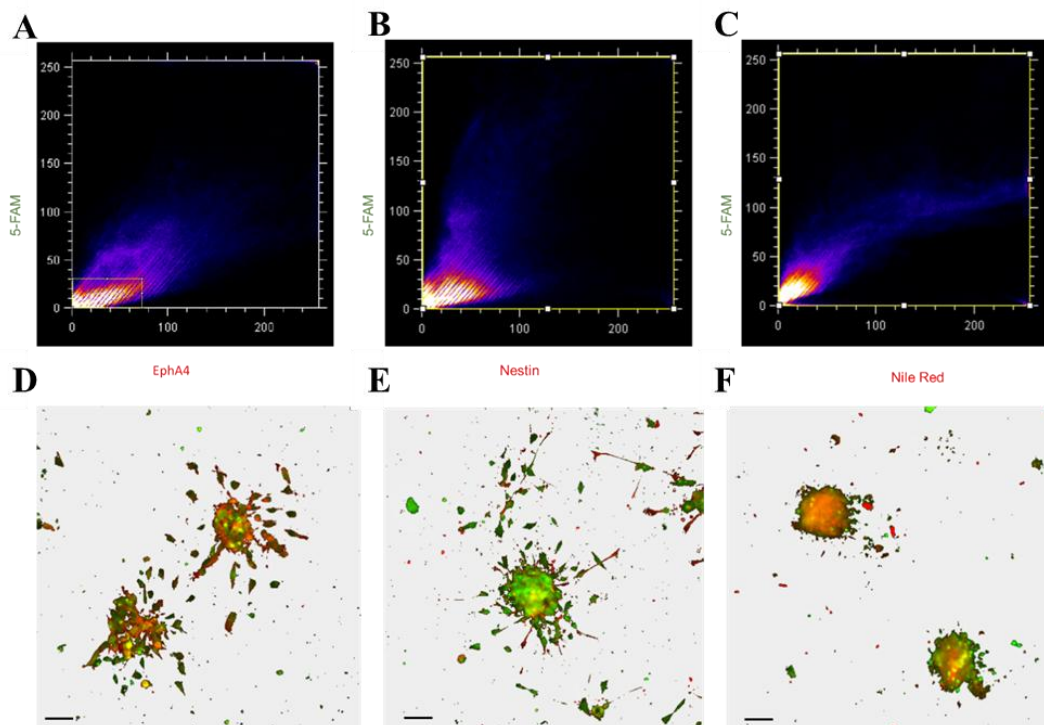


Figure 2.12 Colocalization analysis of 5-FAM-Exo-pep-11 with NSC marker using ImageJ.

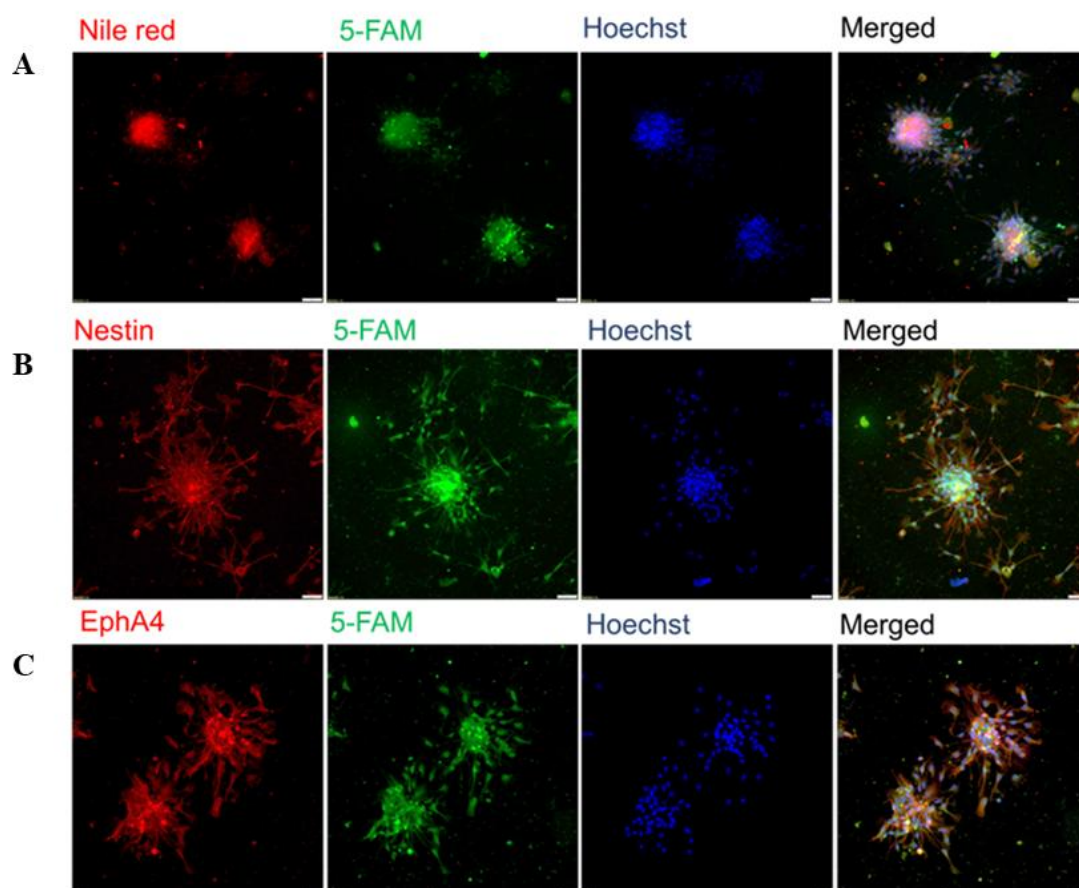


Figure 2.13 Uptake analysis of peptide-functionalized EV in NSC culture. (A) Uptake analysis of 5-FAM peptide conjugated Nile red labeled EV (5-FAM-Exo-Pep-11-nile red). (B) Uptake of 5-FAM peptide conjugated EV (5-FAM-Exo-Pep-11) and EphA4 antibody staining. (C) Uptake of 5-FAM-Exo-Pep-11 and Nestin immunocytochemistry. The scale bar represents 50 μm .

2.4.3 Exo-pep-11 is non-cytotoxic, and shows increased NSC proliferation

To check the Cytotoxicity, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was performed with various concentrations of peptide 11 and Exo-pep-11. The result indicated the peptide and Exo-pep-11 are nontoxic in concentrations as high as 400 μM and 100 $\mu\text{g/mL}$, respectively (Figure 2.14 A, B). Additionally, Exo-pep-11 shows more than 100% cell viability in MTT assay; this can be an indication of the cell proliferative nature of Exo-pep-11. Apart from nontoxic nature Exo-pep-11 shows good serum stability with half-life of approximate 16 hrs (Figure 2.9 D). The NSC proliferative nature of Exo-pep-11 was confirmed using BrdU cell proliferation assay. The result shows upon treatment with 40 $\mu\text{g/mL}$ Exo-Pep-11, the NSC culture shows increased proliferation at 48 hr (Figure 2.14 C) and proliferation increase to ~ 1.9 -fold at 72 hr (Figure 2.14 D). In the control experiment with unmodified EVs, cellular proliferation was lower compared to that observed with Exo-pep-11. This difference may be attributed to the higher cellular uptake of Exo-pep-11 relative to the unmodified EVs (S15). From the result, it is clear that peptide 11 does not have any therapeutic effect; it only helps in receptor-specific uptake, and the therapeutic effect is attributed to EV.

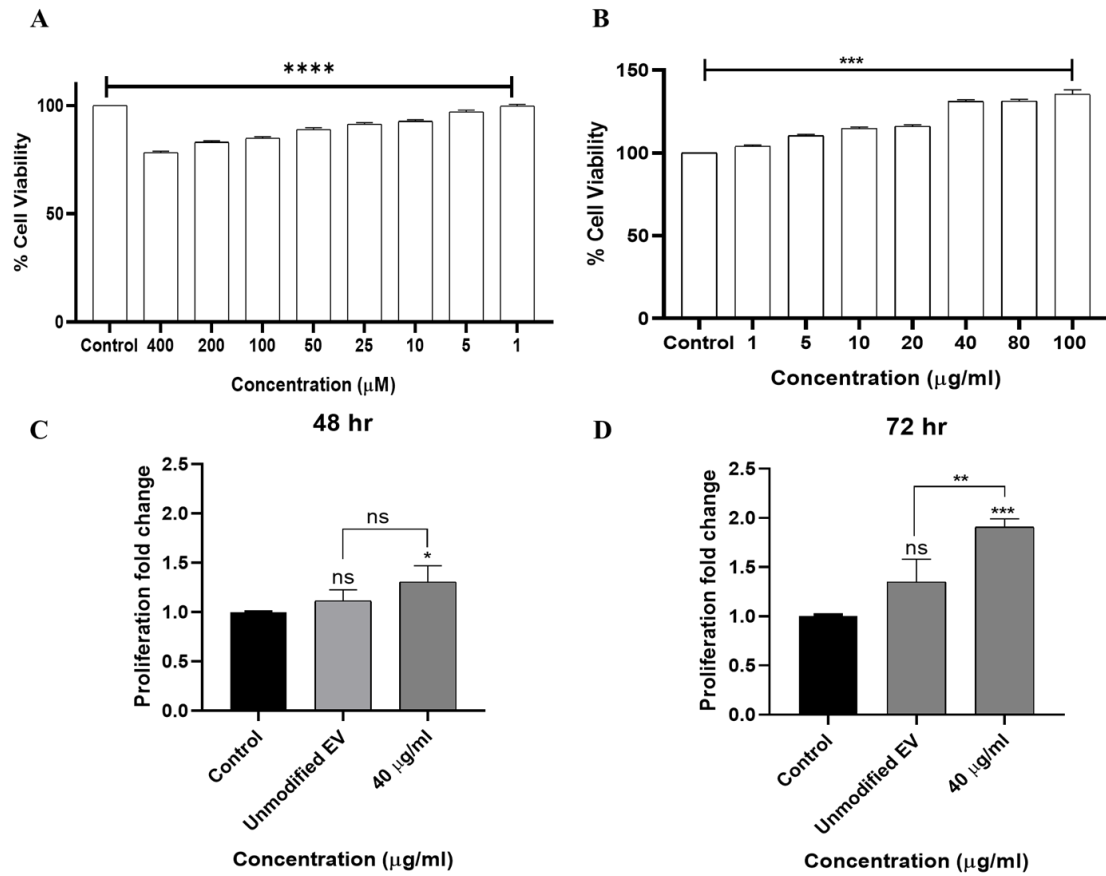


Figure 2.14 Cytotoxicity assessment and Proliferation analysis. (A) Cytotoxicity analysis of Peptide 11 at 400, 200, 100, 50, 25, 10, 5, and 1 μM concentration in NSC culture (number of samples = 3). (B) Cytotoxicity analysis of Exo-Pep-11 at 1, 5, 10, 20, 40, 80, and 100 μg/mL concentration (number of samples = 3). (C) Assessment of cellular proliferation in NSC culture treated with 40 μg/mL Exo-Pep-11 for 48 hr (number of samples = 5). (D) Assessment of cellular proliferation in NSC culture treated with 40 μg/mL Exo-Pep-11 for 72 hr (number of samples = 5). (p-value < 0.05= *, p-value < 0.01= **, p-value < 0.001= ***, p-value < 0.0001 = ****.)

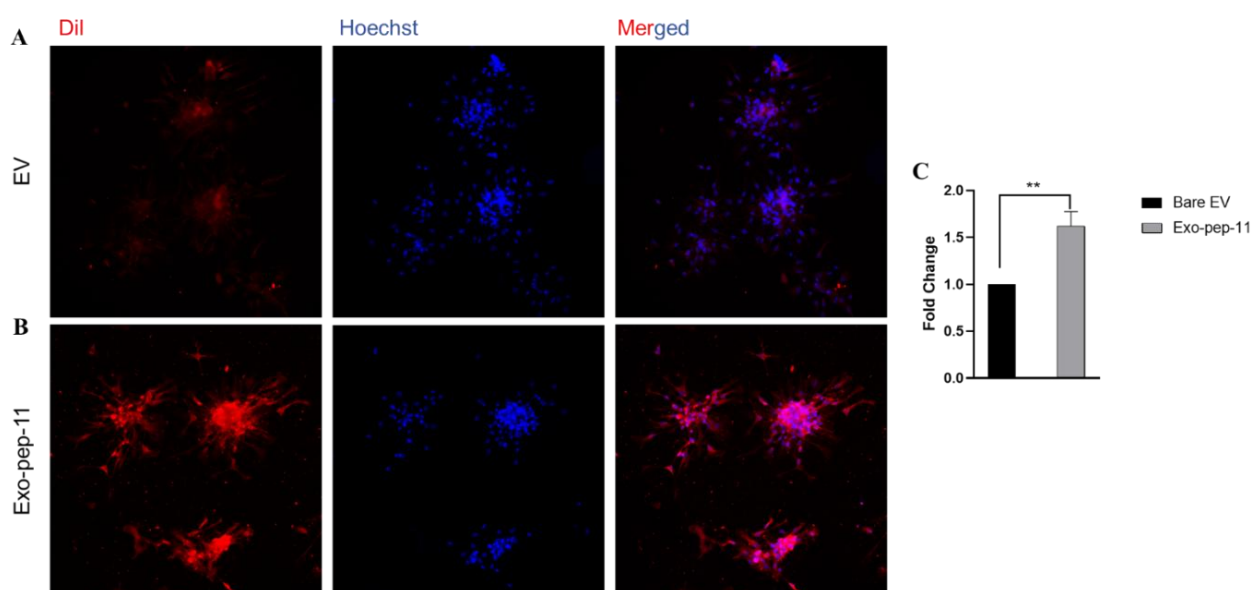


Figure 2.15 Uptake analysis of Dil labeled EV. A) cellular uptake of Dil labeled bare EV B) cellular uptake of Dil labeled Exo-pep-11 C) quantification of cellular uptake of base EV and Exo-pep-11.

2.4.4 Exo-pep-11 treated NSC shows higher expression of ID1 and Nestin

Nestin is a marker for NSC, and it is required for proper self-renewal of NSC ³⁹. From our experimental study (Figure 2.16), we have found there is a decline of Nestin expression in NSC culture isolated from aging rat SVZ (12-13 months old), which upon treatment with 40 $\mu\text{g}/\text{mL}$ of Exo-pep-11 leads to ~1.4-fold increase in Nestin expression (Figure 2.16 A, B). Cells expressing high levels of ID1 are defined as B1 Type Adult Neural Stem Cells ⁴⁰. From our experiment, we have found that the NSC isolated from adult rat SVZ shows a low level of ID1 expression, and upon treatment with 40 $\mu\text{g}/\text{mL}$ of Exo-pep-11, the ID1 expression increased ~2-fold (Figure 2.16 C, D). In cells treated with unmodified EVs, the expression of Nestin and ID1 is lower compared to cells treated with Exo-pep-11. This difference is likely due to the reduced uptake of unmodified EVs relative to Exo-pep-11. This result indicates the NSC isolated from rat SVZ are Nestin and ID1 positive, and Exo-pep-11 helps in the rejuvenation of NSC.

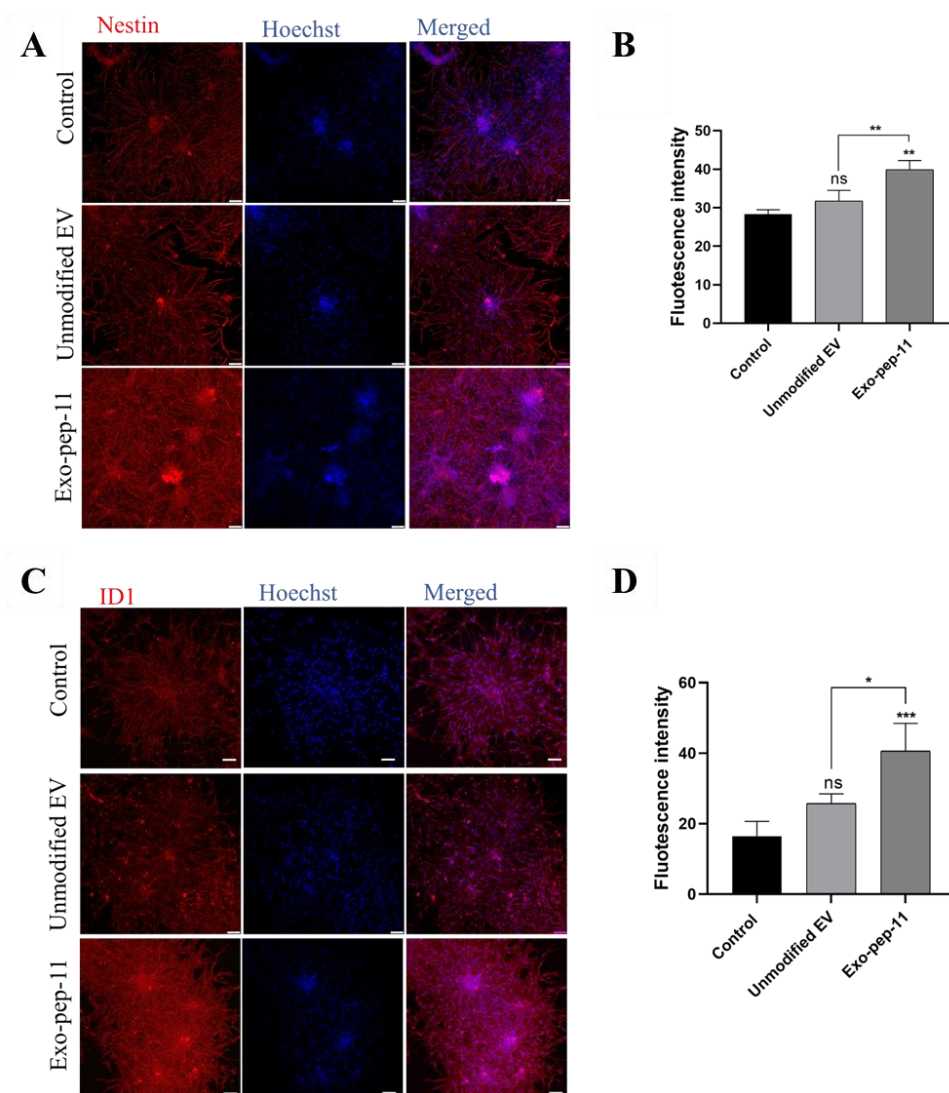


Figure 2.16 NSC marker expression analysis after engineered EV treatment. (A) Nestin immunocytochemistry in the untreated control (upper panel), unmodified EV treated (middle panel), and Exo-pep-11 treated (lower panel) NSC culture (B) Quantification of Nestin expression (number of samples = 10). (C) ID1 immunocytochemistry in untreated (upper panel), unmodified EV treated (middle panel), and Exo-pep-11 treated (lower panel) NSC culture. (D) Quantification of ID1 expression (number of samples = 10). The scale bar represents 100 μ m.

2.4.5 Exo-pep-11 internalized by NSC located in SVZ and colocalize with NSC-specific marker

So far, our experimental data reveals internalization of Exo-Pep-11 by NSC, proliferation activity, and rejuvenation of NSC in the *in vitro* system. Then, we decided to check the therapeutic potential of Exo-Pep-11 in *in vivo* by NSC in *in vivo* aging rats (12-13 months old). To check the NSC targeting property of engineered EV, we have injected 5-FAM-Exo-Pep-11 or 5-FAM-Exo-Pep-11-Nile red (100 μ g in 20 μ L PBS) in the adult rat lateral ventricle using stereotaxic apparatus. We have data similar to those we got in the *in vitro* system. The engineered EV is internalized specifically by NSC present in rat SVZ. The uptake was confirmed using immunohistochemistry and colocalization of 5-FAM-Exo-pep-11 with NSC marker Nestin (Figure 2.17 A) and EphA4 (Figure 2.17 B). 5-FAM-Exo-Pep-11-Nile red is taken up by the same cell and shows a fluorescence signal in both green and red channels; this also

confirms the successful conjugation of peptide and EV (Figure 2.17 C). ImageJ colocalization finder plugin was used to analyze the images to confirm colocalization (Figure 2.18).

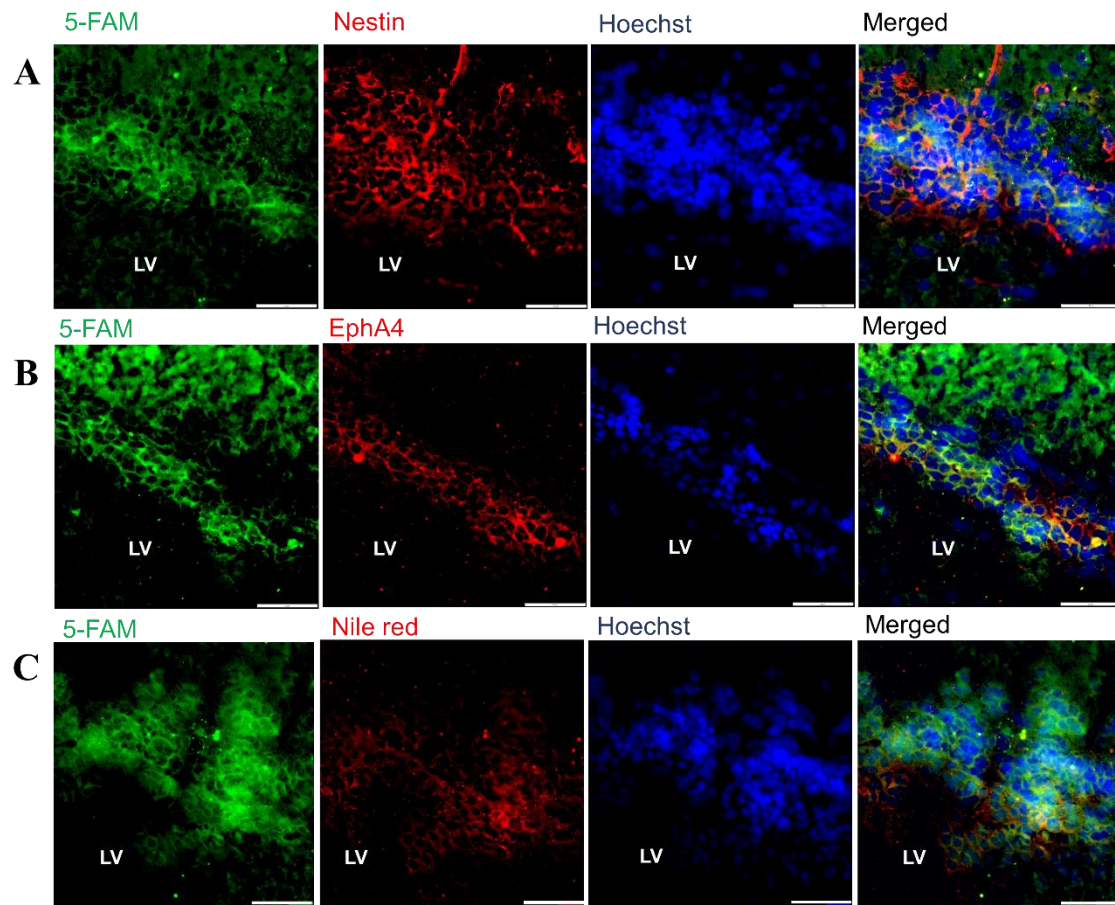


Figure 2.17 Uptake of engineered EV by rat SVZ NSC. (A) Uptake analysis of 5-FAM-Exo-Pep-11 and Nestin immunohistochemistry. (B) Uptake analysis of 5-FAM-Exo-Pep-11 and EphA4 immunohistochemistry. (C) Uptake analysis of 5-FAM-Exo-Pep-11-nile red. The scale bar represents 50 μm.

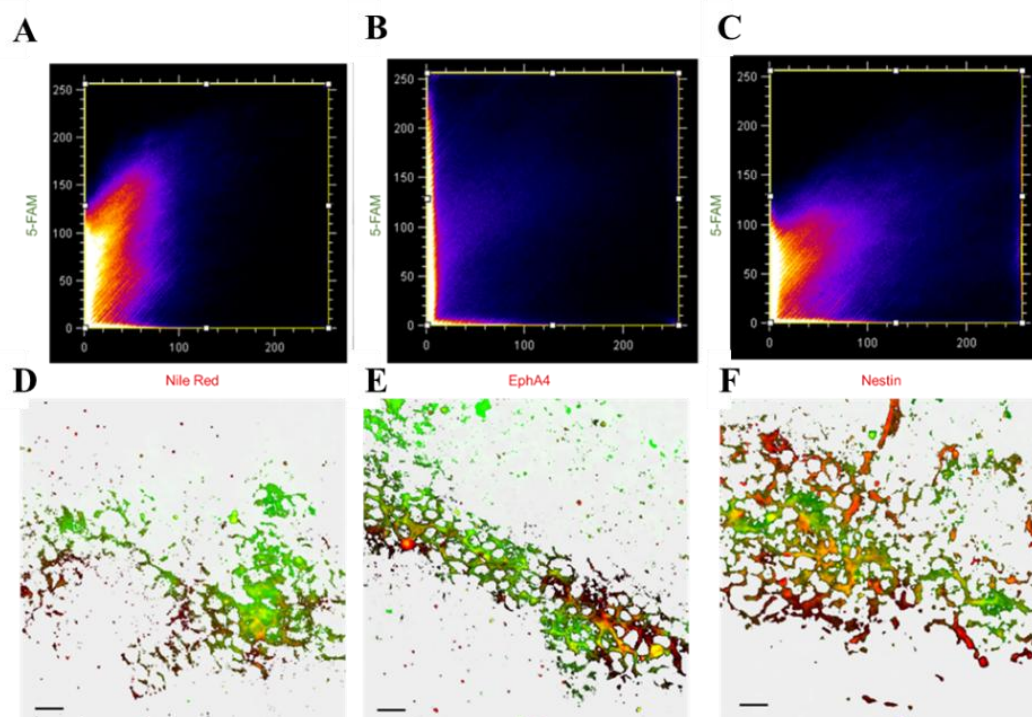


Figure 2.18 Colocalization analysis of 5-FAM-Exo-pep-11 with NSC marker using ImageJ in rat brain slice.

2.4.6 Exo-pep-11 is internalized by NSC in a receptor-specific manner

The above results indicated that Exo-Pep-11 is taken up by NSC in *in vitro* and *in vivo* systems. Next, we conducted a tryptophan quenching assay to assess the binding affinity of Peptide 11 for EphA4. The results revealed a binding affinity of $2.1380 \times 10^4 \text{ M}^{-1}$ (Figure 2.20). Then, we were interested in seeing if NSC takes up the engineered EV in a receptor-specific manner. The peptide-11 was designed to target EphA4; to confirm this, we have conducted two experiments. The first experiments involved the treatment of an NSC culture with EphA4 primary antibody for 30 min before 5-FAM-Exo-Pep-11 treatment, and EphA4 untreated cells were kept as a control. Then, the uptake of 5-FAM-Exo-Pep-11 was analyzed using a fluorescent microscope. The imaging data shows that the uptake of engineered EV was decreased by ~2.3-fold in EphA4 antibody-treated cells as compared to untreated control (Figure 2.19 A, B). This confirms that the uptake is solely EphA4 receptor targeted. The second experiment involved co-immunoprecipitation of Peptide-11 with EphA4 in combinations, listed in Figure 2.19 C. After co-immunoprecipitation, the EphA4 was detected by Dot blot (Figure 2.19 D) and Peptide 11 was detected using ESI-MS. In conditions "a" and "b" the peptide was present in the supernatant as well as in the precipitate, as evident from ESI-MS data (Figure 2.21 A, B). But in condition "c" when the primary antibody is incubated with protein prior to peptide 11, even though the precipitate contains the EphA4 protein, it does not contain peptide-11, which can be seen from dot blot and ESI-MS data, respectively. (Figure 2.21 C). The explanation for the result obtained from condition "c" will be due to the EphA4 primary antibody-mediated blockage of the peptide-11 binding site. In case "d" due to antibody-mediated blockage, we are getting Peptide-11 Mass in the supernatant (Figure 2.21 D), but we are not getting EphA4 in the supernatant; this confirms co-immunoprecipitation without peptide-11. These two results indicate the uptake of Exo-pep-11 is solely EphA4 targeted.

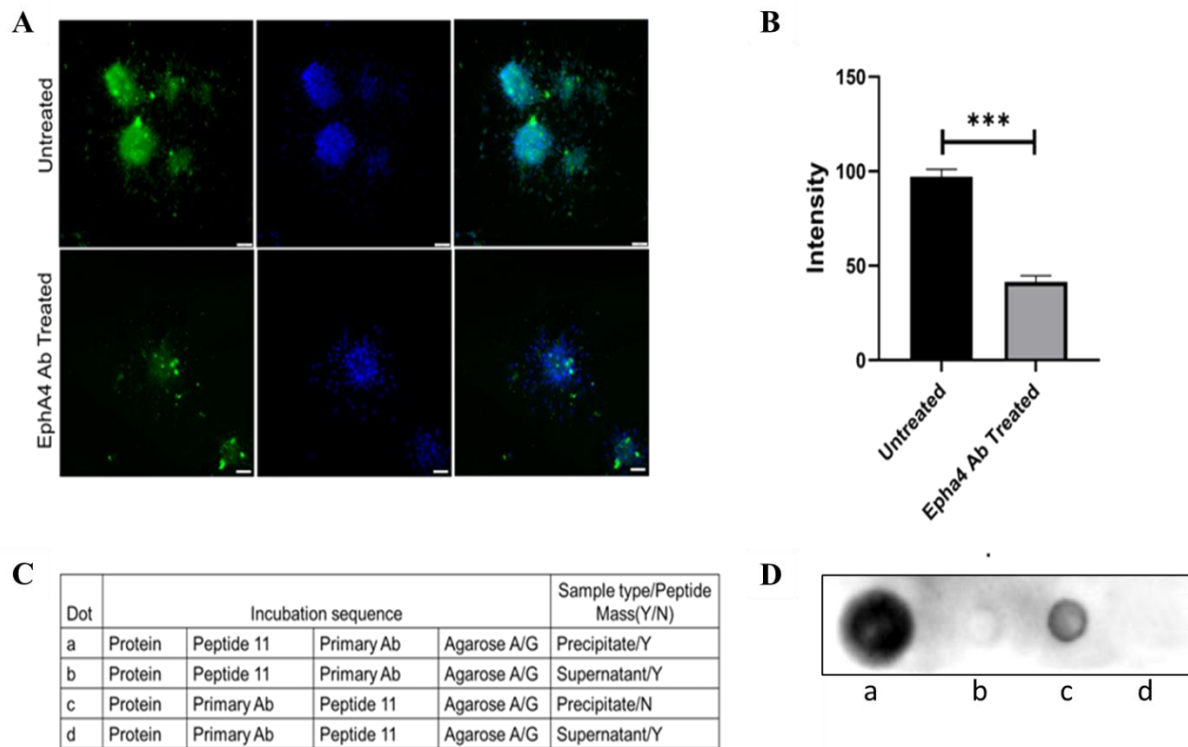


Figure 2.19 Receptor-specific uptake analysis. (A) The upper panel shows cellular uptake in the control cell, and the lower panel shows uptake of engineered EV in the EphA4 antibody-treated cell. The scale bar represents 50 μm . (B) Quantification of uptake analysis in control cell and antibody-treated cell (number of samples = 5). (C) Shows different conditions for immunoprecipitation and subsequent mass spectroscopic analysis results. (D) Dot blot analysis after co-immunoprecipitation.

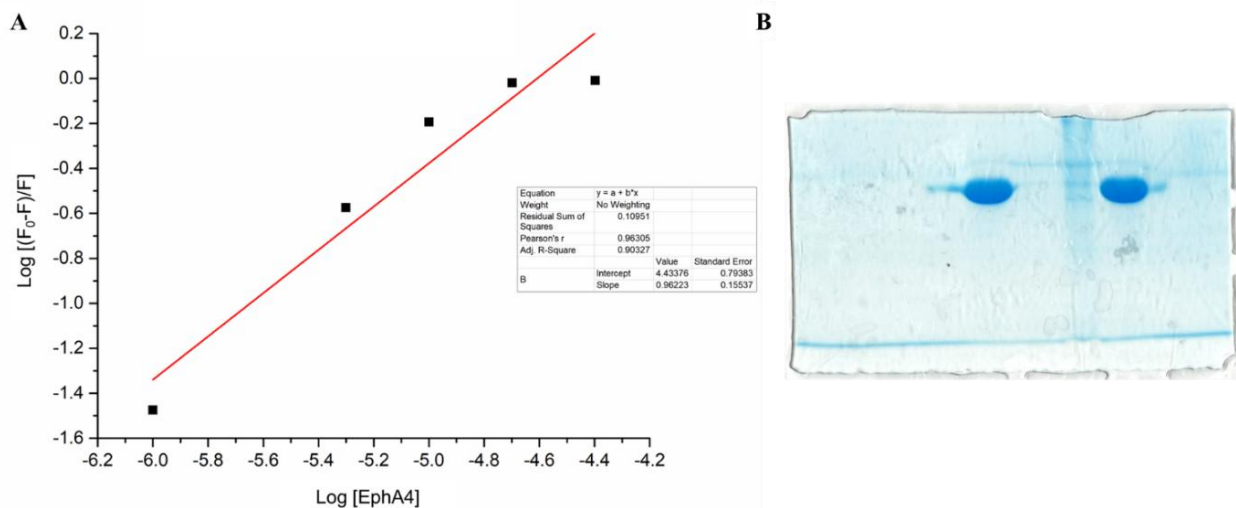


Figure 2.20 A) Standard curve for Tryptophan quenching assay B) Coomassie brilliant blue stained Immunoprecipitated EphA4 loaded gel image.

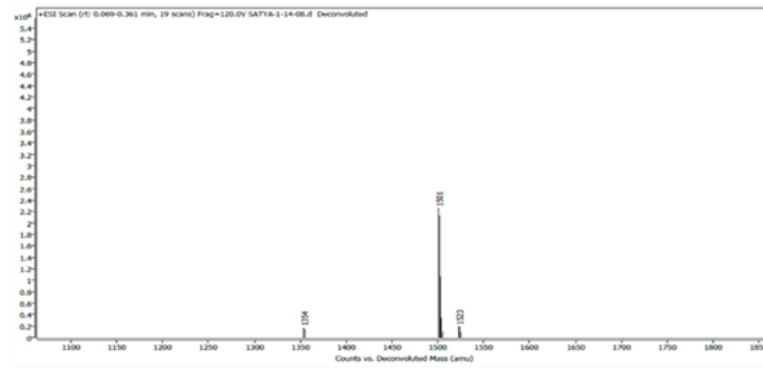
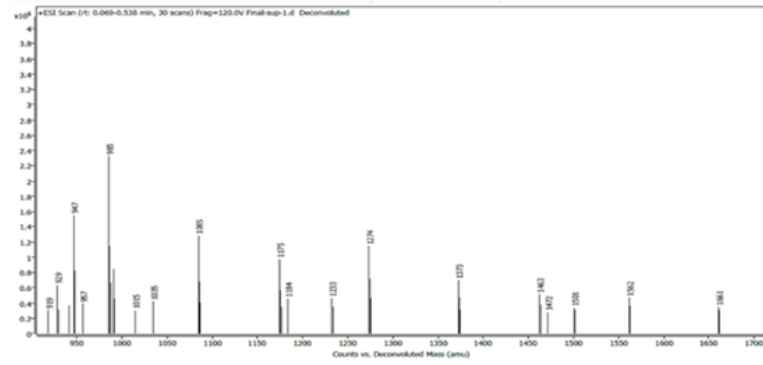
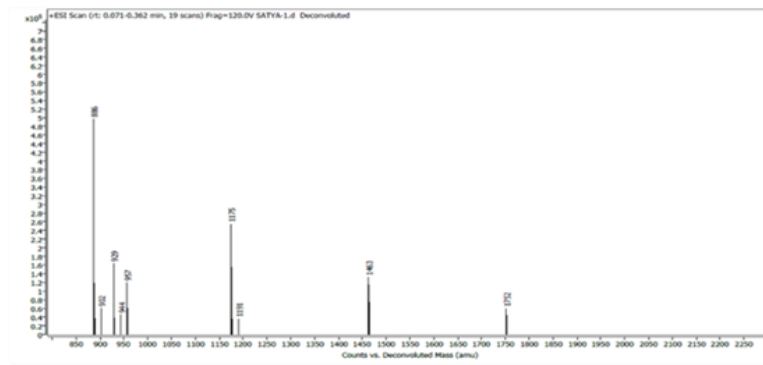
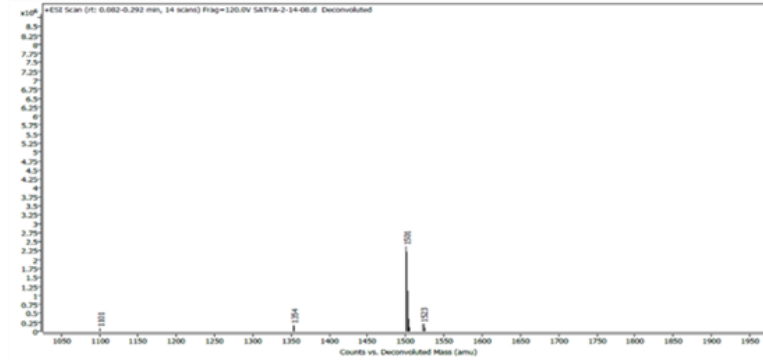
A**B****C****D**

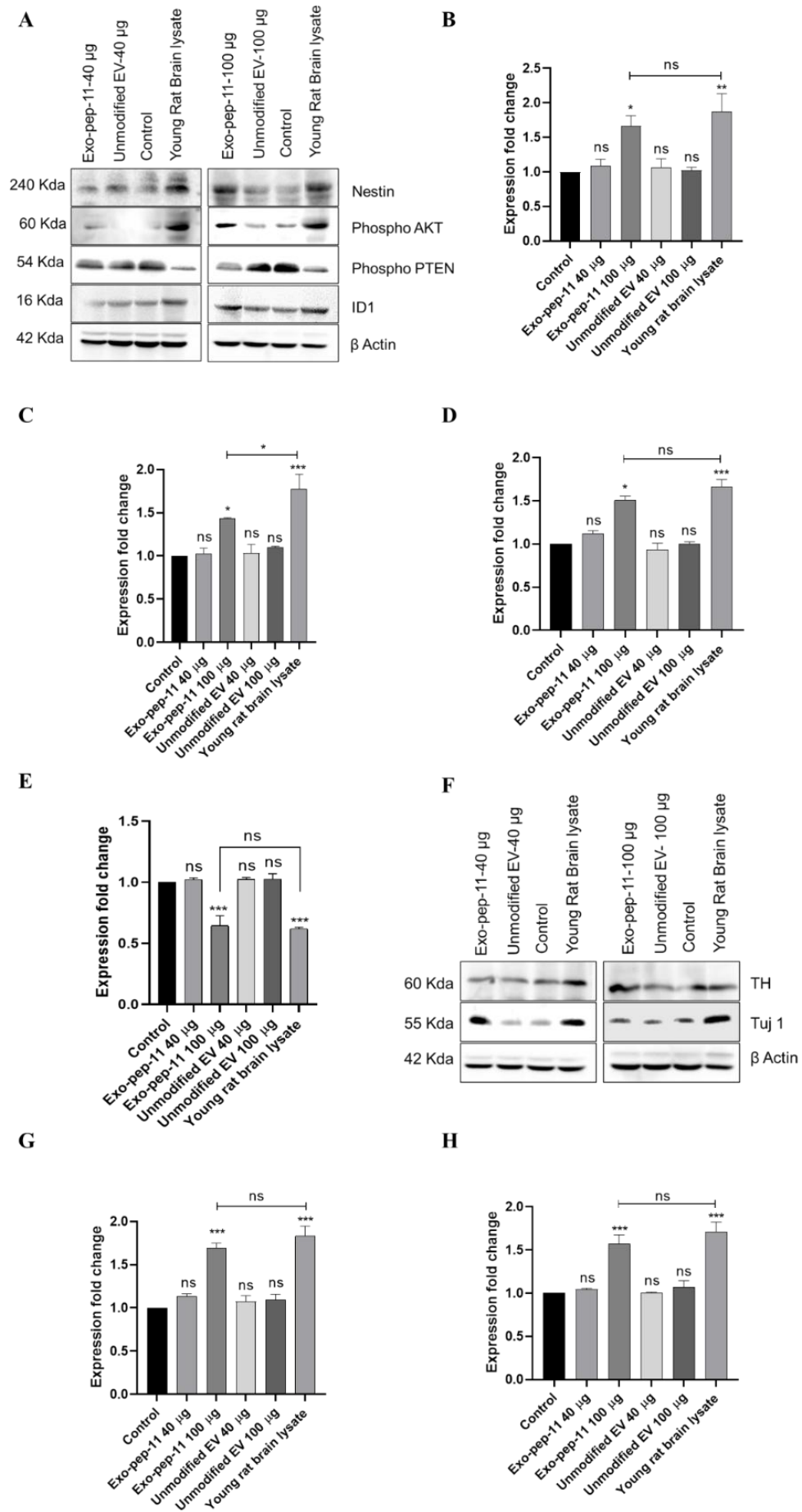
Figure 2.21 Mass Spectroscopic analysis of coimmunoprecipitation assay elute and supernatant.

2.4.7 Exo-pep-11 enhances expression of NSC markers and neurogenic markers in aging rat brain SVZ and olfactory bulb, respectively

From the above-discussed results, it is clear that Exo-pep-11 shows NSC-specific uptake and NSC rejuvenation properties. Then, we were interested in checking the effect of Exo-pep-11 in adult aging rats. After Stereotaxic injection of 40 µg and 100 µg of Exo-pep-11 or 40 µg and 100 µg of Unmodified EVs, some rats were sacrificed after 3 days, and protein was isolated from rat SVZ, and other rats were sacrificed after 7 days, and protein was isolated from rat olfactory bulb. The proteins were then used to check the expression of NSC-specific markers and neurogenic markers by western blot. As we have seen in *in vitro* data, we have similar data in rat brain lysate. In control animals, we have less expression of Nestin and ID1, which confirms the signs of aging in rats. We have got increased expression of Nestin and Id1 in protein isolated from engineered-EV treated rat SVZ as compared to unmodified EV and untreated rat (Figure 2.22 A, B, C). Exo-pep-11 is also nonimmunogenic to rat at concentration as high as 100 µg (Figure 2.22 I). Then, we were interested in the neurogenic potential of Exo-Pep-11. Towards this venture, we have analyzed the expression of Tuj1 and TH in protein isolated from engineered-EV treated rat Olfactory Bulbs. We have found a low level of expression of Tuj1 and TH, which can be an indication of aging-related neuron loss. After Exo-pep-11 treatment, we found a higher expression of neurogenic markers TH and Tuj1 compared to the control and unmodified EV rat Olfactory Bulb (Figure 2.22 F, G, H). These data confirm the dual activity of Exo-pep-11 in NSC rejuvenation and olfactory bulb neurogenesis.

2.4.8 Exo-pep-11 enhances NSC proliferation by enhancing phospho-AKT expression

The above results demonstrate the proliferative nature of Exo-Pep-11. Next, we were interested in finding the pathway of NSC proliferation. As it has been previously reported PTEN negatively regulates neural stem cell self-renewal ⁴¹. Numerous studies have also established the role of PI3K/Akt/PTEN in stem cells. In mouse embryonic stem cells (mESCs) and adult stem cells, the loss of PTEN is correlated with the activation of Akt, leading to increased self-renewal and proliferation ^{41, 42}. So, we were interested in checking the expression of AKT and PTEN (Figure 2.22 A, D, E). From the western blot analysis of the protein isolated from aging rat SVZ, we found increased expression of AKT compared to control and decreased expression of PTEN compared to control. In control, there is an increased expression of PTEN, which inhibits the expression of AKT and results in decreased NSC self-renewal. In the case of Exo-pep-11-treated rats, the decrease in PTEN expression leads to the activation of AKT, which results in increased NSC self-renewal. The results confirm that Exo-pep-11 exerts its NSC self-renewal property via the PTEN/AKT pathway.



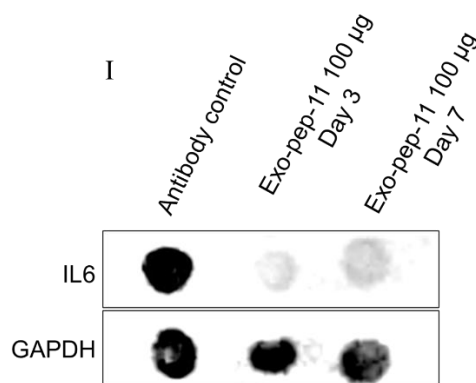


Figure 2.22 Western blot analysis, quantification and immunogenicity assessment (A) Nestin, Phospho AKT, Phospho PTEN, and ID1 expression analyzed in protein isolated from rat SVZ treated with 100 µg Exo-Pep-11 and Exo-Pep-11 untreated control. Quantification of (B) Nestin and (C) ID1 (D) Phospho AKT (E) Phospho PTEN protein expression (Number of samples= 3). (F) TUJ1 and TH expression were analyzed in protein isolated from treated and untreated rat Olfactory Bulbs. Quantification of (G) TH and (H) TUJ1 protein expression (Number of samples= 3). (I) Expression analysis of proinflammatory cytokine IL6 in protein isolated from rat SVZ at day 3 and day 7 of Exo-pep-11 treatment.

2.5 CONCLUSION

In this study, we have designed a library of peptides from the EphA4-Ephrin B2 complex to target the EphA4 receptor present in NSC. After computational library screening and cytotoxicity assessment, we selected Peptide-11 and synthesized the same. This was then functionalized onto the surface of EV-isolated from rat adipose tissue stem cell, the engineered EV here termed Exo-Pep-11. Interestingly, we observed that Exo-pep-11 is nontoxic to cells and taken up by NSC in a receptor-targeted manner in both *in vitro* and *in vivo* conditions. This engineered EV also increases NSC proliferation in aging rat SVZ by activating the PTEN/AKT pathway and increases neurogenesis in aging rat Olfactory Bulb. Additionally, due to the neurogenic potential, this engineered EV can also find its effectiveness in aging-related olfactory dysfunction, which is also one of the earliest non-motor symptoms seen in over 90% of Parkinson's disease patients. Our results indicate that the peptide itself does not possess therapeutic potential; rather, it facilitates targeted delivery of extracellular vesicles (EV), and the demonstrated enhancement in neural stem cell (NSC) proliferation and neurogenesis is attributed to rat adipose tissue-stem cells derived EV. In summary, the amalgamation of a novel functionalization approach and the incorporation of both targeting and therapeutic elements collectively establish the uniqueness of Exo-Pep-11.

2.6 REFERENCE

1. Rakic, P., Adult neurogenesis in mammals: an identity crisis. *J. Neurosci.*, **2002**, 22 (3), 614-618.
2. Alvarez-Buylla, A.; García-Verdugo, J. M. Neurogenesis in adult subventricular zone. *J. Neurosci.*, **2002**, 22 (3), 629-634.
3. Lledo, P.-M.; Alonso, M.; Grubb, M. S., Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci.*, **2006**, 7 (3), 179-193.
4. Alvarez-Buylla, A.; García-Verdugo, J. M.; Tramontin, A. D. A unified hypothesis on the lineage of neural stem cells. *Nat. Rev. Neurosci.*, **2001**, 2 (4), 287-293.
5. García-Verdugo, J. M.; Doetsch, F.; Wichterle, H.; Lim, D. A.; Alvarez-Buylla, A. Architecture and cell types of the adult subventricular zone: in search of the stem cells. *J. Neurobiol.*, **1998**, 36 (2), 234-248.
6. Wichterle, H.; García-Verdugo, J. M.; Alvarez-Buylla, A. Direct evidence for homotypic, glia-independent neuronal migration. *Neuron*, **1997**, 18 (5), 779-791.

7. Kornack, D. R.; Rakic, P. The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc. Natl. Acad. Sci. U.S.A.*, **2001**, 98 (8), 4752-4757.
8. Lois, C.; Garcia-Verdugo, J.-M.; Alvarez-Buylla, A. Chain migration of neuronal precursors. *Science*, **1996**, 271 (5251), 978-981.
9. Lledo, P.-M.; Merkle, F. T.; Alvarez-Buylla, A. Origin and function of olfactory bulb interneuron diversity. *Trends Neurosci.*, **2008**, 31 (8), 392-400.
10. Whitman, M. C.; Greer, C. A. Adult neurogenesis and the olfactory system. *Prog. Neurobiol.*, **2009**, 89 (2), 162-175.
11. Nada, M.-B.; Slomianka, L.; Vyssotski, A. L.; Lipp, H.-P. Early age-related changes in adult hippocampal Neurogenesis in C57 mice. *Neurobiol. Aging*, **2010**, 31 (1), 151-161.
12. Lugert, S.; Basak, O.; Knuckles, P.; Haussler, U.; Fabel, K.; Götz, M.; Haas, C. A.; Kempermann, G.; Taylor, V.; Giachino, C. Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell*, **2010**, 6 (5), 445-456.
13. Encinas, J. M.; Michurina, T. V.; Peunova, N.; Park, J.-H.; Tordo, J.; Peterson, D. A.; Fishell, G.; Koulakov, A.; Enikolopov, G. Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell*, **2011**, 8 (5), 566-579.
14. Bouab, M.; Paliouras, G.; Aumont, A.; Forest-Berard, K.; Fernandes, K. J. Aging of the subventricular zone neural stem cell niche: evidence for quiescence-associated changes between early and mid-adulthood. *Neuroscience*, **2011**, 173, 135-149.
15. Altman, J.; Das, G. D. Autoradiographic and histological evidence of postnatal hippocampal Neurogenesis in rats. *J. Comp. Neurol.*, **1965**, 124 (3), 319-335.
16. Kempermann, G.; Kuhn, H. G.; Gage, F. H. Experience-induced Neurogenesis in the senescent dentate gyrus. *J. Neurosci.*, **1998**, 18 (9), 3206-3212.
17. Kuhn, H. G.; Dickinson-Anson, H.; Gage, F. H. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.*, **1996**, 16 (6), 2027-2033.
18. Seki, T.; Arai, Y. Age-related production of new granule cells in the adult dentate gyrus. *Neuroreport*, **1995**, 6 (18), 2479-2482.
19. Bondolfi, L.; Ermini, F.; Long, J. M.; Ingram, D. K.; Jucker, M. Impact of age and caloric restriction on Neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol. Aging*, **2004**, 25 (3), 333-340.
20. Corenblum, M. J.; Ray, S.; RemLey, Q. W.; Long, M.; Harder, B.; Zhang, D. D.; Barnes, C. A.; Madhavan, L. Reduced Nrf2 expression mediates the decline in neural stem cell function during a critical middle-age period. *Aging cell*, **2016**, 15 (4), 725-736.
21. Jinno, S. Decline in adult neurogenesis during aging follows a topographic pattern in the mouse hippocampus. *J. Comp. Neurol.*, **2011**, 519 (3), 451-466.
22. Enwere, E.; Shingo, T.; Gregg, C.; Fujikawa, H.; Ohta, S.; Weiss, S. Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory Neurogenesis, and deficits in fine olfactory discrimination. *J. Neurosci.*, **2004**, 24 (38), 8354-8365.
23. Maslov, A. Y.; Barone, T. A.; Plunkett, R. J.; Pruitt, S. C. Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J. Neurosci.*, **2004**, 24 (7), 1726-1733.
24. Tropepe, V.; Craig, C. G.; Morshead, C. M.; van der Kooy, D. Transforming growth factor- α null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. *J. Neurosci.*, **1997**, 17 (20), 7850-7859.
25. Luo, J.; Daniels, S. B.; Lenington, J. B.; Notti, R. Q.; Conover, J. C. The aging neurogenic subventricular zone. *Aging cell*, **2006**, 5 (2), 139-152.
26. Haehner, A.; Hummel, T.; Reichmann, H. Olfactory loss in Parkinson's disease. *Parkinson's disease*, **2011**, 450939.
27. Ghosh, S.; Ghosh, S. Exosome: The "Off-the-Shelf" Cellular Nanocomponent as a Potential Pathogenic Agent, a Disease Biomarker, and Neurotherapeutics. *Front. pharmacol.*, **2022**, 13, 878058.
28. Ghosh, S.; Garg, S.; Ghosh, S., Cell-derived exosome therapy: a novel approach to treat post-traumatic brain injury mediated neural injury. *ACS Chem. Neurosci.*, **2020**, 11 (14), 2045-2047.
29. Cui, G. H.; Wu, J.; Mou, F. F.; Xie, W. H.; Wang, F. B.; Wang, Q. L.; Fang, J.; Xu, Y. W.; Dong, Y. R.; Liu, J. R. Exosomes derived from hypoxia-preconditioned mesenchymal stromal cells ameliorate cognitive decline by rescuing synaptic dysfunction and regulating inflammatory responses in APP/PS1 mice. *FASEB J.*, **2018**, 32 (2), 654-668.

30. Zhao, X.; Wu, X.; Qian, M.; Song, Y.; Wu, D.; Zhang, W. Knockdown of TGF- β 1 expression in human umbilical cord mesenchymal stem cells reverts their exosome-mediated EMT promoting effect on lung cancer cells. *Cancer Lett.*, **2018**, 428, 34-44.
31. Moon, G. J.; Sung, J. H.; Kim, D. H.; Kim, E. H.; Cho, Y. H.; Son, J. P.; Cha, J. M.; Bang, O. Y. Application of mesenchymal stem cell-derived extracellular vesicles for stroke: biodistribution and microRNA study. *Transl. Stroke Res.*, **2019**, 10, 509-521.
32. Rao, F.; Zhang, D.; Fang, T.; Lu, C.; Wang, B.; Ding, X.; Wei, S.; Zhang, Y.; Pi, W.; Xu, H. Exosomes from human gingiva-derived mesenchymal stem cells combined with biodegradable chitin conduits promote rat sciatic nerve regeneration. *Stem Cells Int.*, **2019**, 2019.
33. Riazifar, M.; Mohammadi, M. R.; Pone, E. J.; Yeri, A.; Lasser, C.; Segaliny, A. I.; McIntyre, L. L.; Shelke, G. V.; Hutchins, E.; Hamamoto, A. Stem cell-derived exosomes as nanotherapeutics for autoimmune and neurodegenerative disorders. *ACS nano*, **2019**, 13 (6), 6670-6688.
34. Lépinoux-Chambaud, C.; Barreau, K.; Eyer, J. The neurofilament-derived peptide NFL-TBS. 40-63 targets neural stem cells and affects their properties. *Stem Cells Transl. Med.* **2016**, 5 (7), 901-913.
35. Baudet, S.; Bécrot, J.; Nicol, X. Approaches to manipulate Ephrin-A: EphA forward signaling pathway. *Pharmaceutics*, **2020**, 13 (7), 140.
36. Khodosevich, K.; Watanabe, Y.; Monyer, H. EphA4 preserves postnatal and adult neural stem cells in an undifferentiated state in vivo. *J. Cell Sci.*, **2011**, 124 (8), 1268-1279.
37. Mamber, C.; Kozareva, D. A.; Kamphuis, W.; Hol, E. M. Shades of gray: the delineation of marker expression within the adult rodent subventricular zone. *Prog. Neurobiol.*, **2013**, 111, 1-16.
38. Xu, H.; Liao, C.; Liang, S.; Ye, B.-C. A novel peptide-equipped exosomes platform for delivery of antisense oligonucleotides. *ACS Appl. Mater. Interfaces.*, **2021**, 13 (9), 10760-10767.
39. Park, D.; Xiang, A. P.; Mao, F. F.; Zhang, L.; Di, C.-G.; Liu, X.-M.; Shao, Y.; Ma, B.-F.; Lee, J.-H.; Ha, K.-S. Nestin is required for the proper self-renewal of neural stem cells. *Stem cells*, **2010**, 28 (12), 2162-2171.
40. Nam, H.-s.; Benezra, R. High levels of Id1 expression define B1 type adult neural stem cells. *Cell stem cell*, **2009**, 5 (5), 515-526.
41. Groszer, M.; Erickson, R.; Scripture-Adams, D. D.; Dougherty, J. D.; Le Belle, J.; Zack, J. A.; Geschwind, D. H.; Liu, X.; Kornblum, H. I.; Wu, H. PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. *Proc. Natl. Acad. Sci. U.S.A.*, **2006**, 103 (1), 111-116.
42. Korkaya, H.; Paulson, A.; Charafe-Jauffret, E.; Ginestier, C.; Brown, M.; Dutcher, J.; Clouthier, S. G.; Wicha, M. S. Regulation of mammary stem/progenitor cells by PTEN/Akt/ β -catenin signaling. *PLoS Biol.*, **2009**, 7 (6), e1000121.
43. Kruger, D. M.; Gohlke, H. DrugScorePPI webserver: fast and accurate in silico alanine scanning for scoring protein-protein interactions. *Nucleic Acids Res.*, **2010**, 38 (suppl_2), W480-W486.
44. Faure, G.; Bornot, A.; de Brevern, A. G. Protein contacts, inter-residue interactions and side-chain modelling. *Biochimie*, **2008**, 90 (4), 626-639.
45. Trott, O.; Olson, A. J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.*, **2010**, 31 (2), 455-461.
46. Abraham, M. J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*, **2015**, 1, 19-25.
47. Kaur, A.; Goyal, B. Modelling, Identification of new pentapeptides as potential inhibitors of amyloid- β 42 aggregation using virtual screening and molecular dynamics simulations. *J. Mol. Graph.*, **2023**, 108558.
48. Mukherjee, N.; Ghosh, S.; Sarkar, J.; Roy, R.; Nandi, D.; Ghosh, S. Interfaces, Amyloid-Inspired Engineered Multidomain Amphiphilic Injectable Peptide Hydrogel— An Excellent Antibacterial, Angiogenic, and Biocompatible Wound Healing Material. *ACS Appl. Mater. Interfaces.*, **2023**, 15 (28), 33457-33479.
49. Kassem, D. H.; Habib, S. A.; Badr, O. I.; Kamal, M. M. Isolation of rat adipose tissue mesenchymal stem cells for differentiation into insulin-producing cells. *J. Vis. Exp.*, **2022**, (186), e63348.
50. Théry, C.; Amigorena, S.; Raposo, G.; Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol.*, **2006**, 30 (1), 3.22. 1-3.22. 29.
51. Görgens, A.; Corso, G.; Hagey, D. W.; Jawad Wiklander, R.; Gustafsson, M. O.; Felldin, U.; Lee, Y.; Bostancioglu, R. B.; Sork, H.; Liang, X. Identification of storage conditions stabilizing extracellular vesicles preparations. *J. Extracell. Vesicles*, **2022**, 11 (6), e12238.

52. Walker, T. L.; Kempermann, G. One mouse, two cultures: Isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. *J. Vis. Exp.*, **2014**, (84), e51225.
53. Lee, H. K.; Finniss, S.; Cazacu, S.; Xiang, C.; Brodie, C.; development, Mesenchymal stem cells deliver exogenous miRNAs to neural cells and induce their differentiation and glutamate transporter expression. *Stem cells and dev.*, **2014**, 23 (23), 2851-2861.
54. Belhadj, Z.; Qie, Y.; Carney, R. P.; Li, Y.; Nie, G. Current advances in non-viral gene delivery systems: Liposomes versus extracellular vesicles. *BMEMat*, **1** **2023**, 1 (2), e12018.
55. Armstrong, J. P.; Holme, M. N.; Stevens, M. M. Re-engineering extracellular vesicles as smart nanoscale therapeutics. *ACS nano*, **2017**, 11 (1), 69-83.
56. Han, M.; Yang, H.; Lu, X.; Li, Y.; Liu, Z.; Li, F.; Shang, Z.; Wang, X.; Li, X.; Li, J. Three-dimensional-cultured MSC-derived exosome-hydrogel hybrid microneedle array patch for spinal cord repair. *Nano Lett.*, **2022**, 22 (15), 6391-6401.