



Open Access

Research Article

Expression of Neuron Specific Neuronal Nuclei Protein (NeuNNP) by AAV2 Shows Diagnosis Ability in Spinal Cord Injury Using Neural Stem Cells

Prithiv Kumar KR^{1,2*}, Albert Alukkal^{3,4}

¹Director, Principle Scientist- Poichyadical Stem Cell Centre for Research and Development (POSCERD), Chicago, USA

²CEO, La'zer Group of Companies, Chicago, USA

³MD, Chief Medical Officer- La'zer Healthcare, Chicago, USA

⁴MD, Junior Scientist- Poichyadical Stem Cell Centre for Research and Development (POSCERD), Chicago, USA

***Corresponding Author:** Prithiv Kumar KR, Director, Principle Scientist- Poichyadical Stem Cell Centre for Research and Development (POSCERD), Chicago, USA and CEO, La'zer Group of Companies, Chicago, USA; Email: prithivkumar@gmail.com

Received Date: 16-06-2021; **Accepted Date:** 01-07-2021; **Published Date:** 08-07-2021

Copyright © 2021 by Kumar PKR, et al. All rights reserved. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Abstract

Fibroblast Growth Factor (FGF) shows promising therapy for Spinal Cord Injury (SCI). Neural stem transplantation has been on the role implementing tactics to repair SCI. Considering the hostile hypoxia condition in SCI, Adeno Associated Virus 2 (AAV2) a prototype to AAV is being used for most trials. Basic FGF gene was used in that hostile environment, results showed improved functional recovery in SCI. The regulation of this gene FGF is transduce to yield AAV2-5HRE-bFGF. The improved scores on inclined plane test of BBB (Basso-Bettie-Bresnahan) scale and footprint analysis of functional recovery when compared with vehicle control of AAV2 treatment showed inclination in NeuNNP, neuromodulin GAP43 and neurofilament NF200 and declination in Glial Fibrillary Acidic Protein (GFAP). Other expressions such as LC3-II and Beclin 1 and autophagy-associated proteins all show decline,

Kumar PKR | Volume 2; Issue 2 (2021) | JRMBR-2(2)-016 | Research Article

Citation: Kumar PKR, et al. Expression of Neuron Specific Neuronal Nuclei Protein (NeuNNP) by AAV2 Shows Diagnosis Ability in Spinal Cord Injury Using Neural Stem Cells. J Reg Med Biol Res. 2021;2(2):1-14.

DOI: <http://dx.doi.org/10.46889/JRMBR.2021.2202>

P62 protein is the one that has increased in AAV2 treatment group. This is the key to future treatments of the expression in SCI.

Keywords

Spinal Cord; Neurons; Fibroblast Growth Factor; Lesion Cavity

Introduction

Spinal cord injury changes the structure and function of bone, putting a huge burden on individuals leading to high morbidity and mortality [1]. Central neurons are non-reproducible, therapies such as physical, drug and rehabilitation have significant efficacy for recovery of functions of injured nerves. Neural stem cells have the ability to differentiate multi-directionally and also possess self-renewal techniques which is a useful tool to cure SCI. However this method has few hurdles to tackle, microcirculation disorder, local edema, ischemia and hypoxia that are series of secondary injury. Few other complications like hypoxia environment of SCI, pathogenic autophagy of neuron cells cause deterioration in SCI [2]. Autophagy is the process in which cells wrap the unwanted proteins or damaged organelles into double membrane structure and lead them to lysosomes. It plays an important role in development, differentiation and survival of NSCs. But there are drawbacks to excess of autophagy, causes abnormal proteins in the cells and impairment of normal activities of the cells. Autophagy is induced by variety of stress stimuli, including hypoxia, endoplasmic reticulum stress and DNA damage. Also mitochondria damage is another possibility. There are possibilities of hindering effective repair of nerve cells after SCI. Autophagy was also involved in cell death especially in neurons and spinal cord glial cells after SCI.

Below is the figure for autophagy in spinal cord (Fig. 1) [3].

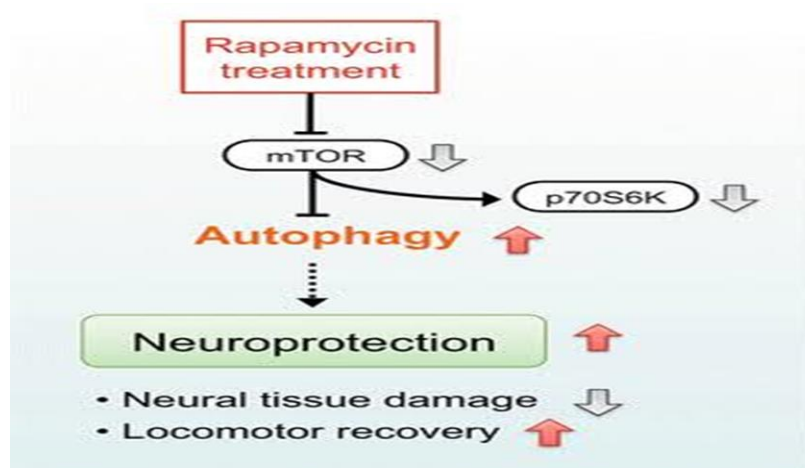


Figure 1: Autophagy in spinal cord.

Kumar PKR | Volume 2; Issue 2 (2021) | JRMBR-2(2)-016 | Research Article

Citation: Kumar PKR, et al. Expression of Neuron Specific Neuronal Nuclei Protein (NeuN) by AAV2 Shows Diagnosis Ability in Spinal Cord Injury Using Neural Stem Cells. J Reg Med Biol Res. 2021;2(2):1-14.

DOI: <http://dx.doi.org/10.46889/JRMBR.2021.2202>

Therapeutic Interventions

The therapeutic interventions of SCI involves neurotrophic factors to reduce damage caused by excessive autophagy in spinal cord, and protect secondary injury, exposure of free radicals and misfolded proteins [4]. There are two pathways classically existing, one regulating autophagy pathway, with mTOR pathways assisted by PI3k-ATK and Phosphate and Tensin Homolog (PTEN) phosphate and Beclin 1- Vps-34 complex pathway. Below is the figure for therapeutic intervention in spinal cord [4].

Beclin-1 over-expresses itself in the membrane associated form of microtubule related protein light chain, cell death happens. The marker used is LC3-II to track autophagy. It is an important signal tool in autophagy process (Fig. 2) [5].

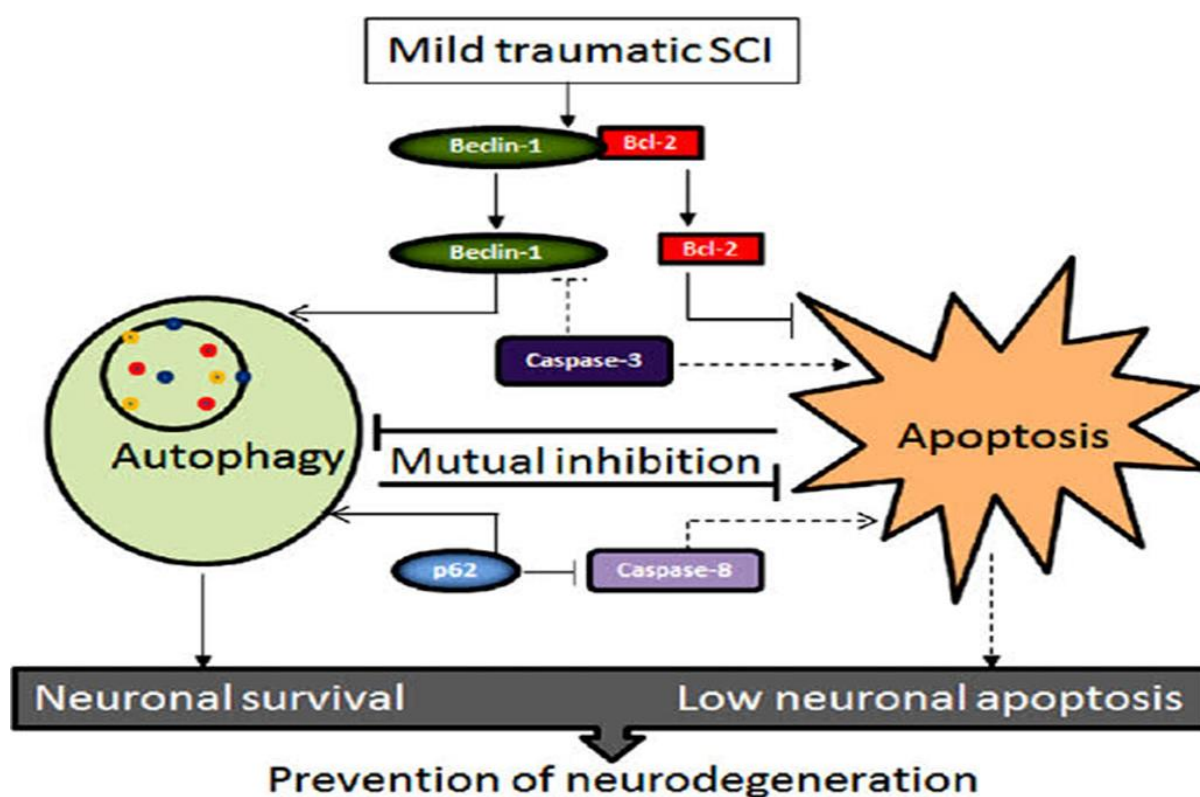


Figure 2: The marker used is LC3-II to track autophagy.

Secondary Process

Another candidate molecule helpful for the SCI repair is basic Fibroblast Growth Factor (bFGF). Its role is repair and recover spinal cord injury whenever deployed one major hurdle it suffers when nerve cells does not survive in hostile hypoxia conditions, only cells that survive them may repair SCI [6]. This system of repair peddles future research and may make it

interesting to study. Below and above images shows recovery of spinal cord after incorporating bFGF (Fig. 3 and 4) [7].

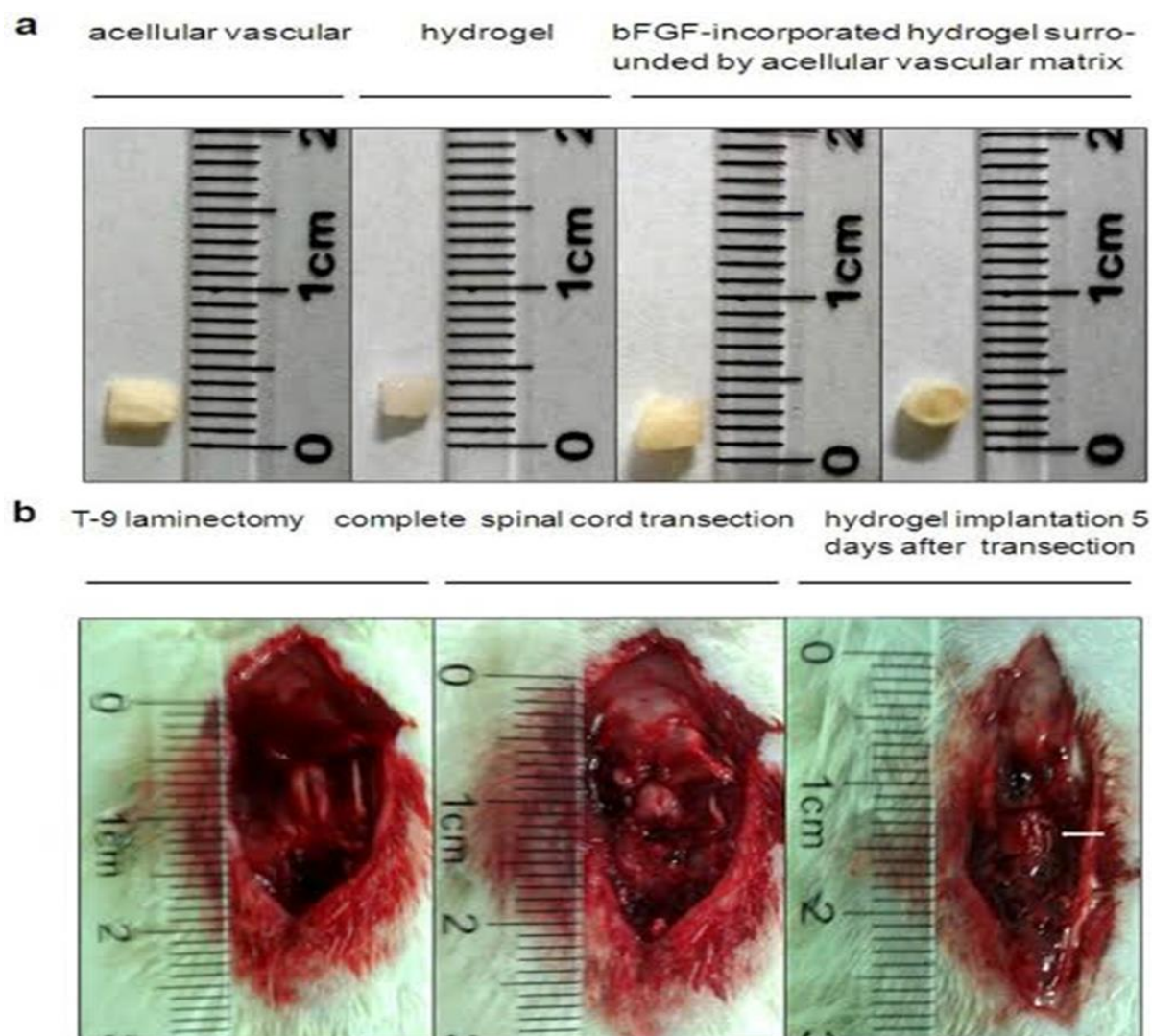


Figure 3: Recovery of spinal cord after incorporating bFGF.

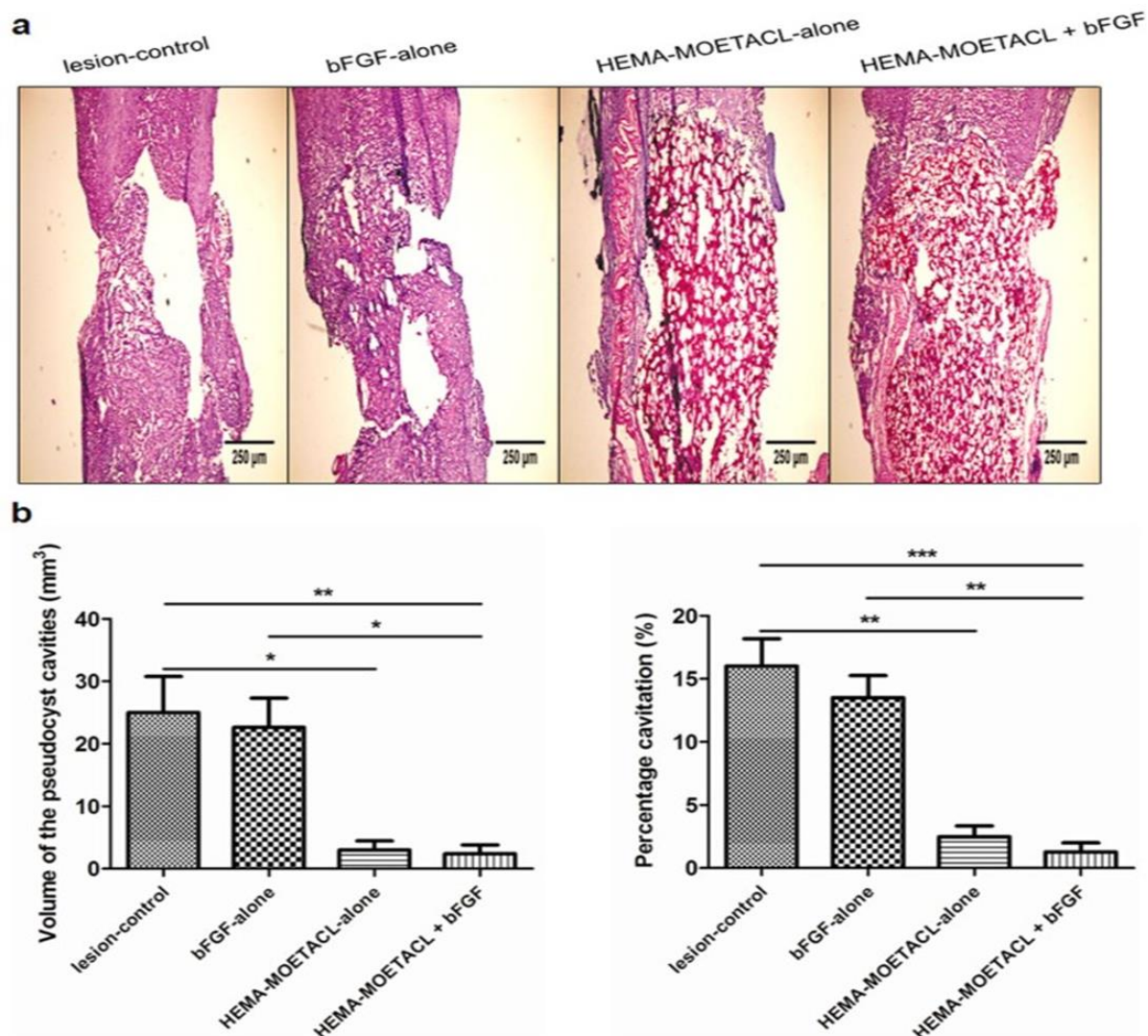


Figure 4: Recovery of spinal cord after incorporating bFGF.

Further Study

In the above study, Hypoxia Response Element (HRE) is employed to control the bFGF combined with a vector known as Adeno Associated Virus (AAV). This vector has neural cell tropism, hence sites like AAV-SHRE-bFGF-NSCs on SCI were generated using array of analysis such as inclined plane test and Basso-Bettie-Bresnahan (BBB) scale, foot print and video recording analysis [8]. The data received shows fruitful effects of the above generated sequence has therapeutic effects on repair of SCI. Below is the microscopic image of analysis [9].

Management of Fibrioblast in SCI

Below image shows management of FGF in SCI (Fig. 5) [9].

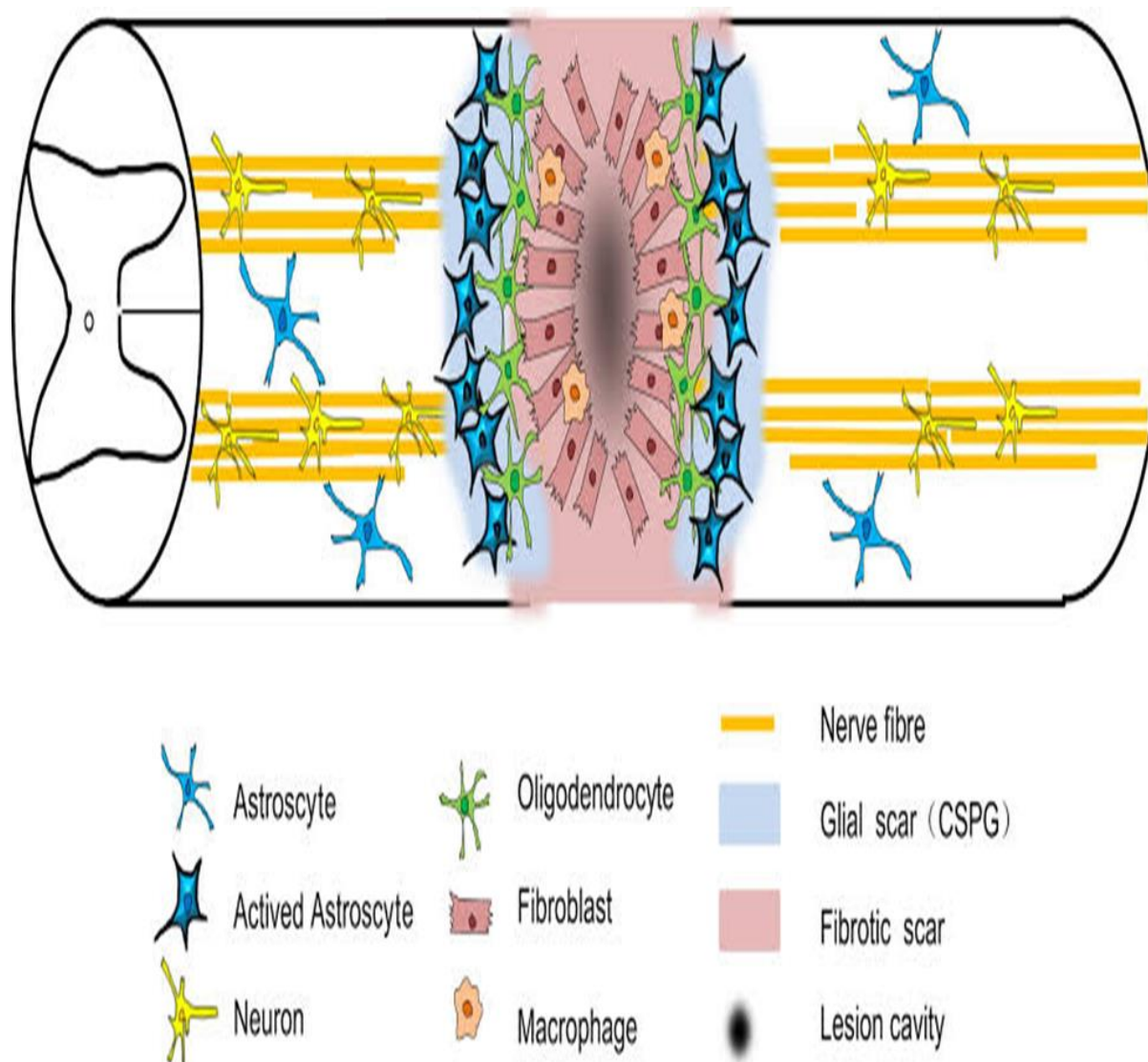


Figure 5: Management of FGF in SCI.

Spinal cord injury is analysed via lesion cavity. Before proceeding further picture of normal spinal cord and injured spinal cord will highlight facts (Fig. 6) [10].

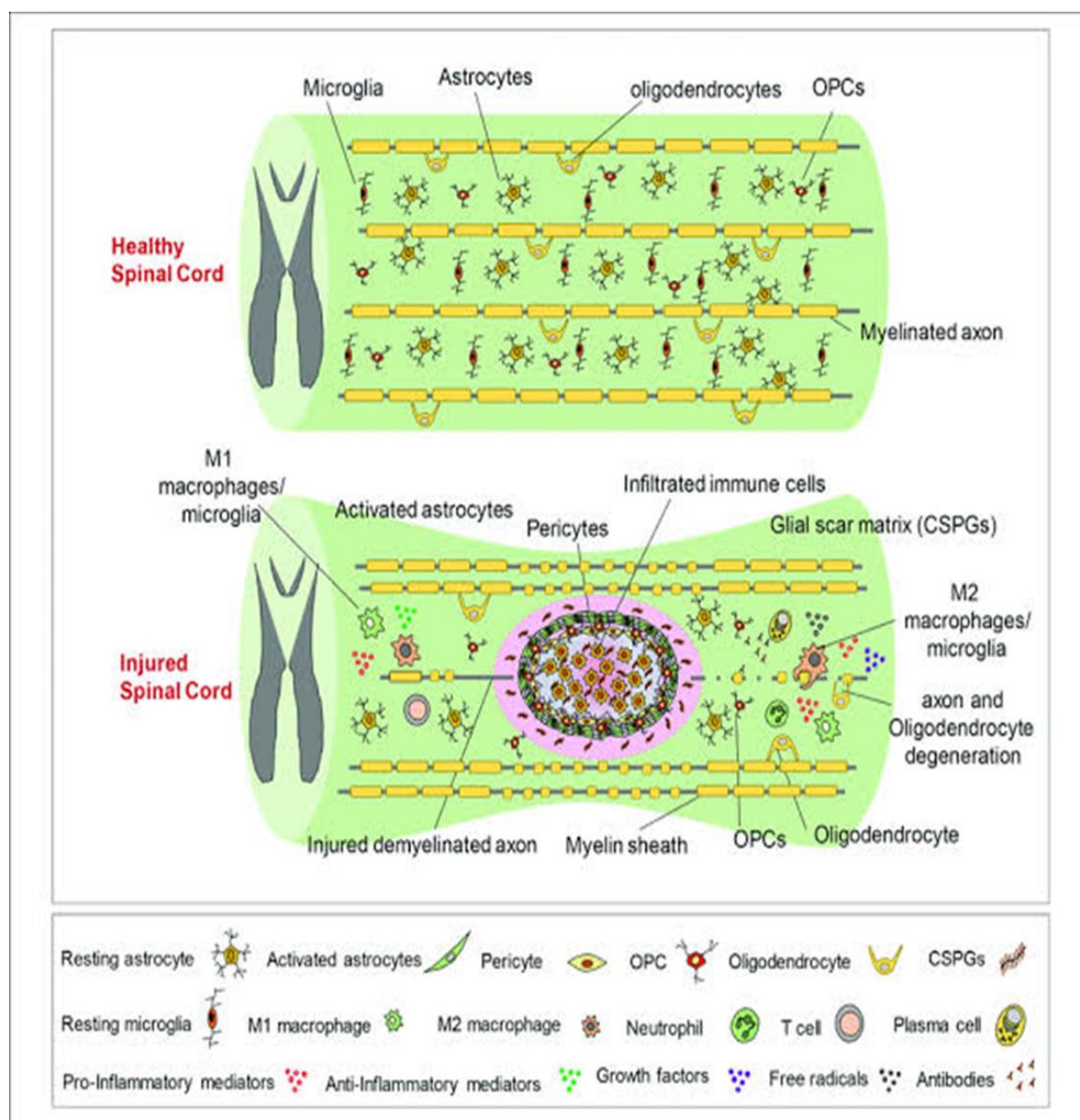


Figure 6: Spinal cord injury analysed.

SCI Model in Rat

Dawley rats were made unconscious with 5% isoflurane and then 3% of same formula before surgery. The T9-T10 laminoid spinous area was removed and exposed. Then the median line was hammered in T9 segment of spinal cord from a height 25 mm to make SCI model.

Below is the rat SCI model (Fig. 7) [11].

After the SCI exposure, it was sutured and put through laminotomy but did not undergo heavy blow procedure. Rats were then put in cage after regular injection of cefazolin sodium [12].

All animal experiments are approved by animal care and national health. They are performed under guidelines of animal care and health. The embryo of Dawley rats aged 14-17 were isolated and cell cultured in Dulbeccos's modified medium added with bFGF (20 mg/ml) and Epidermal Growth Factor (EGF) and 5% cell incubator. This procedure help the NSCs to divide into ~150 balls of nerve cells in culture plate. They are then verified using nestin protein expression by immunofluorescence staining. They are subjected to RAPA for 13 h and sent into observation for finding the effects of autophagic pathway [13].

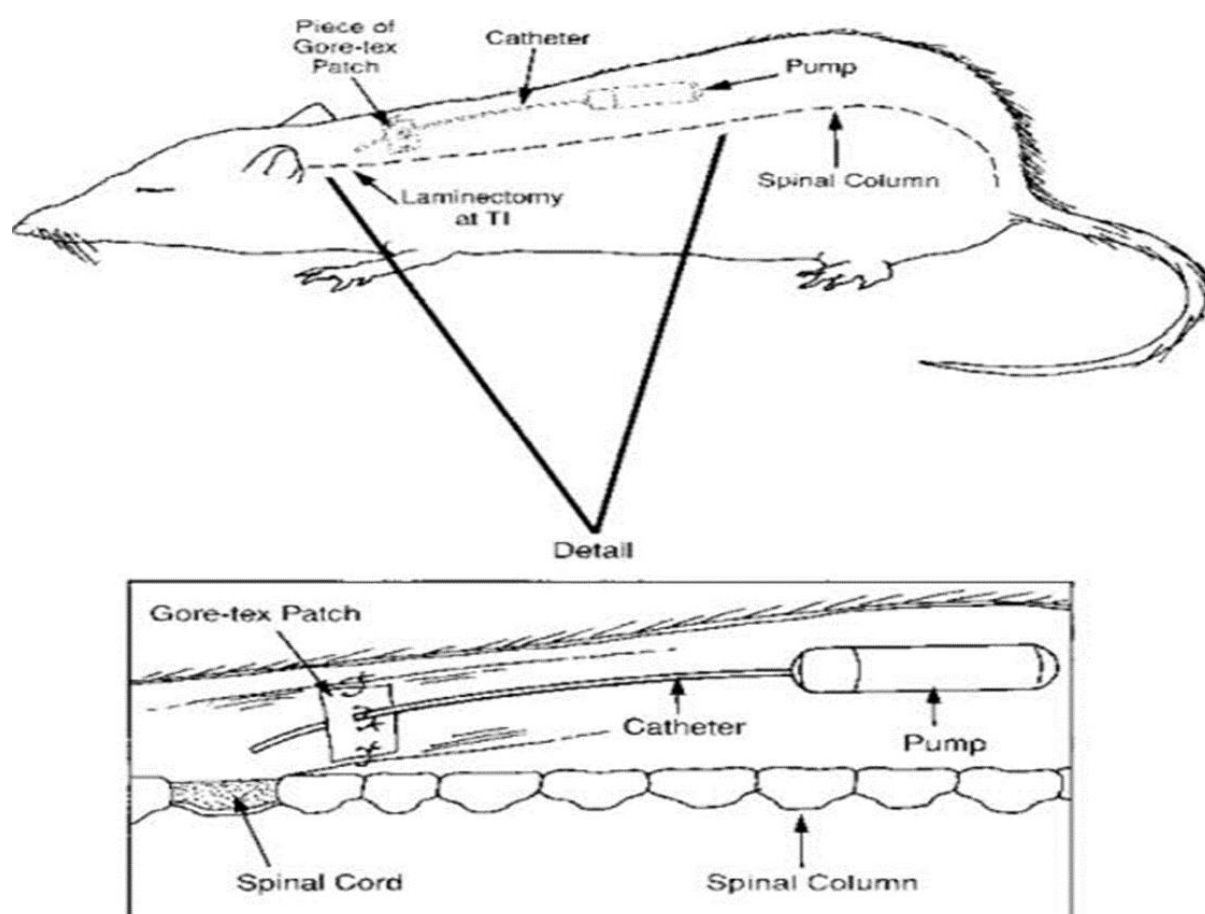


Figure 7: Rat SCI model.

AAV2 Mediated HRE Fronted bFGF Gene Expression

The vectors AAV2-SHRE-bFGF and AAV2-SHRE are generated from pAOV001 pAAV-CAG-MCS backbone plasmid in cloning site Mlu I and Hind II. The SHRE vector is the five repeats of HRE DNA sequence, paced upstream of CMV-cytomegalovirus minimum promoter.

Kumar PKR | Volume 2; Issue 2 (2021) | JRMBR-2(2)-016 | Research Article

Citation: Kumar PKR, et al. Expression of Neuron Specific Neuronal Nuclei Protein (NeuNNP) by AAV2 Shows Diagnosis Ability in Spinal Cord Injury Using Neural Stem Cells. J Reg Med Biol Res. 2021;2(2):1-14.

DOI: <http://dx.doi.org/10.46889/JRMBR.2021.2202>

The vectors were co-transfected and propagated in HEK293 cells. They are purified by caesium chloride quantitative polymerase chain reaction. The final titer of these are found to somewhere at 2.15×10^{14} and 8.12×10^{12} mL/virus particles respectively and employs subsequent NSCs transduction [14]. Further on these transduced cells were subjected to 1×10^3 Multiplicity of Infection (MOI) of vectors. These have a high rate of transduction which attains upto 95%. They are later cultivated in normal culture condition, supernatant protein concentration were detected using bFGF Enzyme Linked Immunosorbent Assay (ELISA). Figure below AAV2 induced procedure (Fig. 8) [15].

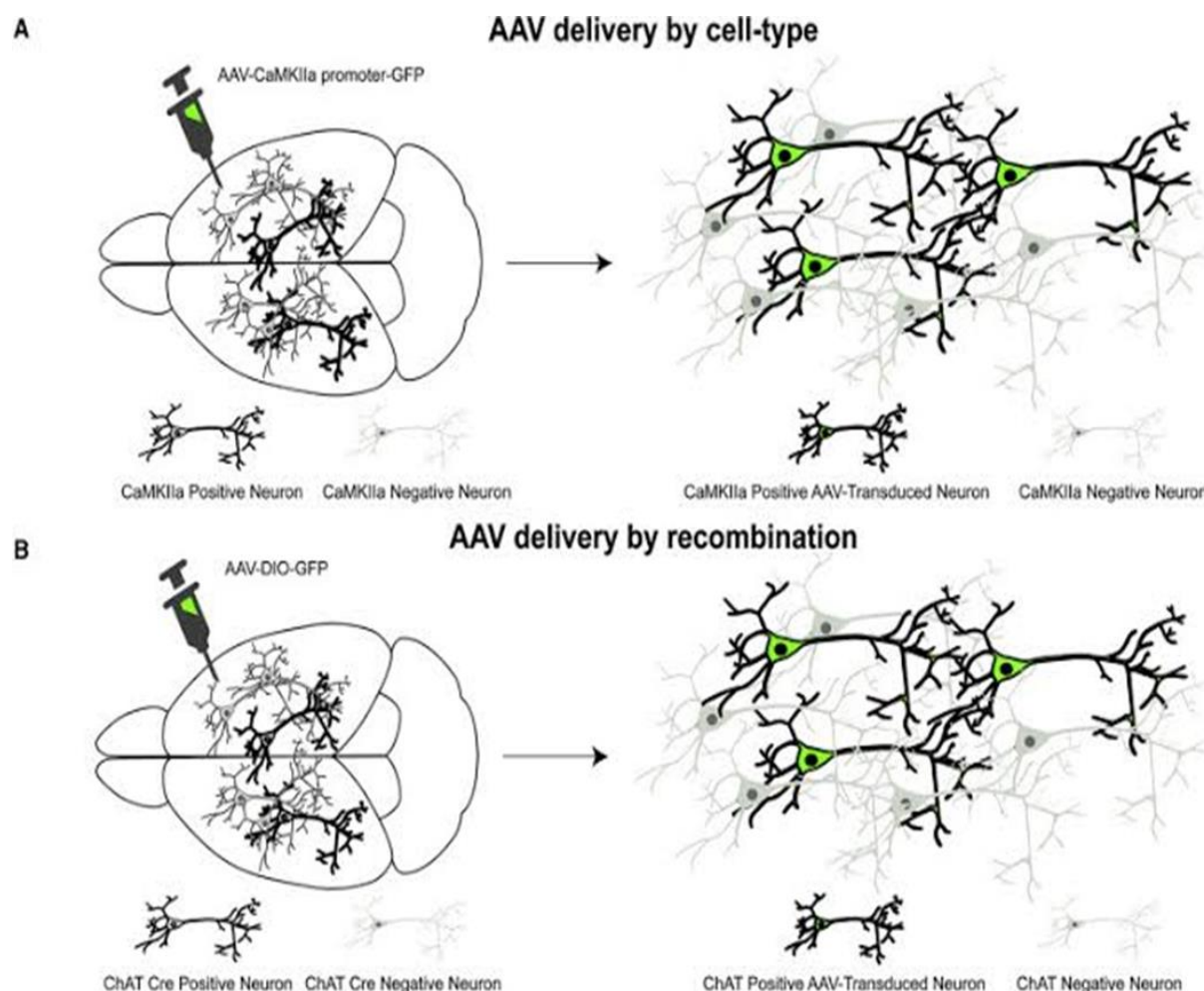


Figure 8: AAV2 induced procedure.

Transport and Replace of NSCs

Dawley rats were subjected to different divisions of each [12]. According to vectors and injury. NSCs were then induced with MOI of AAV2-SHRE-bFGF virus particle for 48 hr. After that

the spinal cord was exposed with 1×10^6 resuspended in 5 nanol of PBS in SCI centre. At different times for 10 different points the recovery was observed [16].

Recovery of SCI and its Recovery Results

Generally recovery is observed through BBB score, the oblique plate test, footprint and video recording images. Conferring the expression of bFGF gene in NSCs they were transplanted to site of SCI rats. AAV2-SHRE NSCs were used as vehicle control. Recovery were observed in 48 hr apart for two months after NSCs replacement. The oblique plate test yielded result to AAV2-SHRE-bFGF-NSCs group of rats named group 1 (Fig. 9). The other tests also showed an imprinting results. The graph was wave shaped and dragged movement. Below is the result of test, observed at different days [17].

The limb movement were also recorded and observed. There were no paralysis seen and movement was horizontal and straight. The group 1 rats showed they can balance the support of body of whole limb [18].

The group 1 rats has normally the lowest maximum height off the ground and footstep error to the highest degree. The group 1 rats showed improvement in limb motor ability after transplantation. Below shows fix of SCI with graph (Fig. 9) [19].

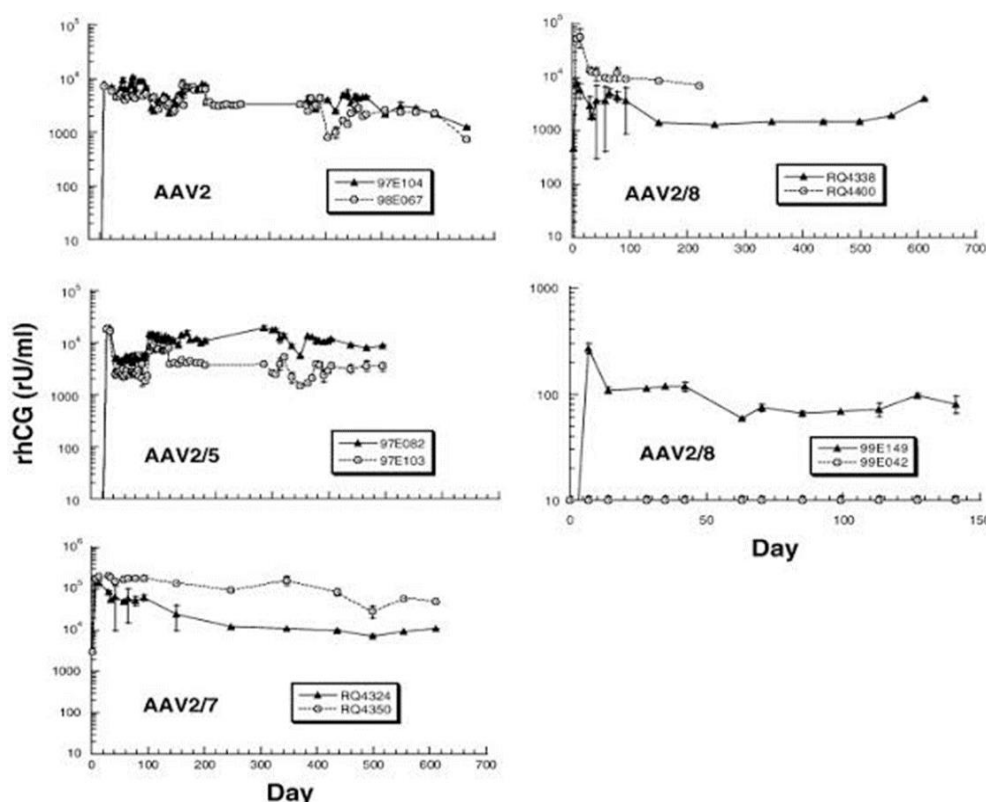


Figure 9: Fix of SCI with graph.

NeuN and GAP43 Work

NeuN an RNA binding protein and neuromodulin GAP43 proteins help in regulating neural generation. After exposing to immunofluorescence staining these NeuN and GAP43 displayed green expression of group 1 rats [20]. On further test with western blot analysis group 1 rats had higher protein expression than all other groups. To enhance the study on group rats for movement of autophagy earlier kept under observation, the autophagy pathway was examined by immunofluorescence staining, result is that both LC3-II and Beclin I cells were decreased and 062 cells were increased. The overall result showed the group 1 rats had better recovery function and also attributed to SCI induced autophagy by AAV2-SHRE-NGF-NSCs [21]. To confirm more these were subjected to RAPA, agonist of autophagy and showed LC3-II was suppressed (Fig. 10) [22].

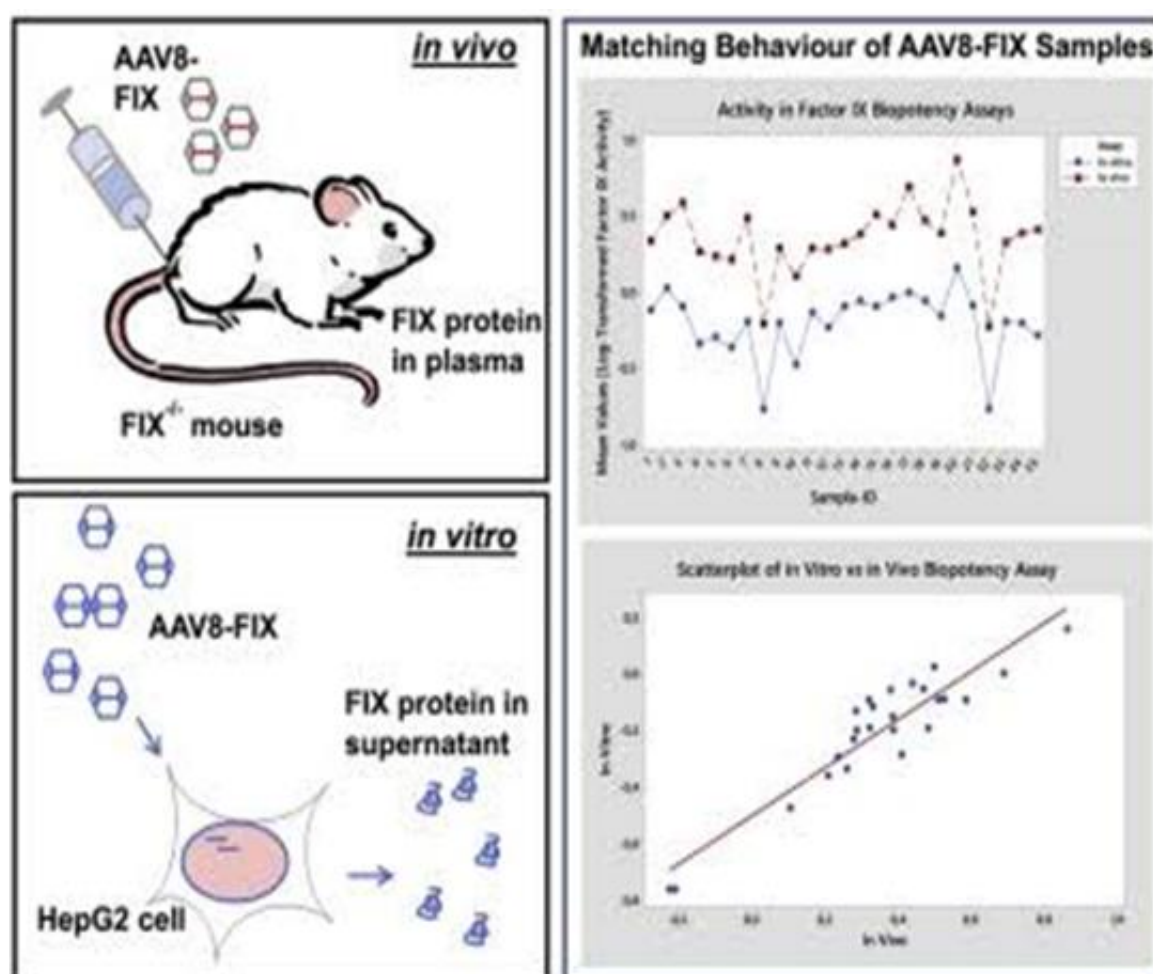


Figure 10: SCI induced autophagy by AAV2-SHRE-NGF-NSCs. To confirm more these were subjected to RAPA, agonist of autophagy and showed LC3-II was suppressed.

Discussion and Conclusion

At cellular level, NSCs replace damaged neurons, oligodendrocytes and astrocytes to promote axon regeneration and myelin sheath reconstruction [23]. At molecular level they release neurotropic factors that help in reconstructing spinal cord. But sometimes the unfavourable microenvironment leads to death of cells or apoptosis of transplanted cells [24]. Also NSCs alteration if ambient oxygen level disturbs the therapy to SCI. The results have shown autophagic death of neurons and glial cells play important role in SCI induced damage and autophagy inhibition may serve therapeutic strategy for SCI [25]. P62 expression played a major role in binding protein complex located inside autophagy activity. They also showed the capacity to accumulate cytoplasm. Moreover Beclin 1 and LC3 II protein levels were raised during autophagy and showed increased P62 expression in group 1 rats [26].

bFGF a key neurotrophic factor in growth of neurons shows attenuated pathological changes in nerve trauma reducing the oxygen radicals antagonising calcium that is overloaded and decreasing the toxicity of nitric oxides. The use AAV2 in temporarily expressing bFGF protein paves the way for future research [27-34].

References

1. Kumar N. Traumatic spinal cord injuries. *The Lancet*. 2011;377(9770):972-4.
2. Fan B, Wei Z, Yao X, Shi G, Cheng X, Zhou X, et al. Microenvironment imbalance of spinal cord injury. *Cell Transplantation*. 2018;27(6):853-66.
3. Taoka Y, Okajima K. Spinal cord injury in the rat. *Prog Neurobiol*. 1998;56:341-58.
4. Silva NA, Sousa N, Reis RL, Salgado AJ. From basics to clinical: a comprehensive review on spinal cord injury. *Progress in neurobiology*. 2014;114:25-57.
5. Kumamaru H, Kadota K, Adler AF, Takashima Y, Graham L, Coppola G, et al. Generation and post-injury integration of human spinal cord neural stem cells. *Nature Methods*. 2018;15(9):723-31.
6. Iwai H, Shimada H, Nishimura S, Kobayashi Y, Itakura G, Hori K, et al. Allogeneic neural stem/progenitor cells derived from embryonic stem cells promote functional recovery after transplantation into injured spinal cord of nonhuman primates. *Stem Cells Trans Med*. 2015;4(7):708-19.
7. Kim HJ, Oh JS, An SS, Pennant WA, Gwak SJ, Kim AN, et al. Hypoxia-specific GM-CSF-overexpressing neural stem cells improve graft survival and functional recovery in spinal cord injury. *Gene Ther*. 2012;19(5):513-21.
8. Gaudin A, Yemisci M, Eroglu H, Lepetre-Mouelhi S, Turkoglu OF, Dönmez-Demir B, et al. Squalenoyl adenosine nanoparticles provide neuroprotection after stroke and spinal cord injury. *Nat Nanotechnol*. 2014;9(12):1054-62.
9. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature*. 2006;441(7097):1094-6.
10. Snyder EY, Teng YD. Stem cells and spinal cord repair. *New Eng J Med*. 2012;366(20):1940-2.
11. Bankston AN, Forston MD, Howard RM, Andres KR, Smith AE, Ohri SS, et al. Autophagy is essential for oligodendrocyte differentiation, survival, and proper myelination. *Glia*. 2019;67(9):1745-59.
12. Halder SK, Kant R, Milner R. Chronic mild hypoxia promotes profound vascular remodeling in spinal cord blood vessels, preferentially in white matter, via an $\alpha 5 \beta 1$ integrin-mediated mechanism. *Angiogenesis*. 2018;21(2):251-66.
13. Cully M. Ion transporter agonist improves recovery. *Nat Rev Drug Discovery*. 2018;17(9):621.

14. Zhang HY, Wang ZG, Wu FZ, Kong XX, Yang J, Lin BB, et al. Regulation of autophagy and ubiquitinated protein accumulation by bFGF promotes functional recovery and neural protection in a rat model of spinal cord injury. *Mol Neurobiol.* 2013;48(3):452-64.
15. Feng J, Chen Y, Lu B, Sun X, Zhu H, Sun X. Autophagy activated via GRP78 to alleviate endoplasmic reticulum stress for cell survival in blue light-mediated damage of A2E-laden RPEs. *BMC Ophthalmol.* 2019;19(1):1-9.
16. He Z, Zhou Y, Huang Y, Wang Q, Zheng B, Zhang H, Li J, et al. DL-3-n-butylphthalide improves functional recovery in rats with spinal cord injury by inhibiting endoplasmic reticulum stress-induced apoptosis. *Am J Transl Res.* 2017;9(3):1075.
17. Zhang H, Wu F, Kong X, Yang J, Chen H, Deng L, et al. Nerve growth factor improves functional recovery by inhibiting endoplasmic reticulum stress-induced neuronal apoptosis in rats with spinal cord injury. *J Transl Med.* 2014;12(1):1-5.
18. Liu S, Sarkar C, Dinizo M, Faden AI, Koh EY, Lipinski MM, et al. Disrupted autophagy after spinal cord injury is associated with ER stress and neuronal cell death. *Cell Death Dis.* 2015;6(1):e1582.
19. Rong Y, Liu W, Wang J, Fan J, Luo Y, Li L, et al. Neural stem cell-derived small extracellular vesicles attenuate apoptosis and neuroinflammation after traumatic spinal cord injury by activating autophagy. *Cell Death Dis.* 2019;10(5):1-8.
20. Fang B, Li XQ, Bao NR, Tan WF, Chen FS, Pi XL, et al. Role of autophagy in the bimodal stage after spinal cord ischemia reperfusion injury in rats. *Neurosci.* 2016;328:107-16.
21. Hu J, Han H, Cao P, Yu W, Yang C, Gao Y, et al. Resveratrol improves neuron protection and functional recovery through enhancement of autophagy after spinal cord injury in mice. *Am J Transl Res.* 2017;9(10):4607-16.
22. Hetz C, Saxena S. ER stress and the unfolded protein response in neurodegeneration. *Nat Rev Neurol.* 2017;13(8):477-91.
23. Grootjans J, Kaser A, Kaufman RJ, Blumberg RS. The unfolded protein response in immunity and inflammation. *Nat Rev Immunol.* 2016;16(8):469-84.
24. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov.* 2013;12:703-19.
25. Hetz C, Axten JM, Patterson JB. Pharmacological targeting of the unfolded protein response for disease intervention. *Nat Chem Biol.* 2019;15(8):764-75.
26. Jeon YJ, Khelifa S, Ratnikov B, Scott DA, Feng Y, Parisi F, et al. Regulation of glutamine carrier proteins by RNF5 determines breast cancer response to ER stress-inducing chemotherapies. *Cancer Cell.* 2015;27(3):354-69.
27. Yu P, Wilhelm K, Dubrac A, Tung JK, Alves TC, Fang JS, et al. FGF-dependent metabolic control of vascular development. *Nature.* 2017;545(7653):224-8.
28. Hu HW, Li XK, Zheng RY, Xiao J, Zeng JQ, Hou ST. bFGF expression mediated by a hypoxia-regulated adenoviral vector protects PC12 cell death induced by serum deprivation. *Biochem Biophys Res Comm.* 2009;390(1):115-20.
29. Fandel TM, Trivedi A, Nicholas CR, Zhang H, Chen J, Martinez AF, et al. Transplanted human stem cell-derived interneuron precursors mitigate mouse bladder dysfunction and central neuropathic pain after spinal cord injury. *Cell Stem Cell.* 2016;19(4):544-57.
30. Liu WG, Wang ZY, Huang ZS. Bone marrow-derived mesenchymal stem cells expressing the bFGF transgene promote axon regeneration and functional recovery after spinal cord injury in rats. *Neurol Res.* 2011;33(7):686-94.
31. Plow EB, Fehlings MG. Hypoxic locomotor rehabilitation for incomplete spinal cord injury: not an oxymoron. *Neurol.* 2014;82:98-9.
32. Sun X, Liu XZ, Wang J, Tao HR, Zhu T, Jin WJ, et al. Changes in neurological and pathological outcomes in a modified rat spinal cord injury model with closed canal. *Neural Reg Res.* 2020;15(4):697.
33. Wang P, Lin C, Wu S, Huang K, Wang Y, Bao X, et al. Inhibition of autophagy is involved in the protective effects of ginsenoside Rb1 on spinal cord injury. *Cellular Mol Neurobiol.* 2018;38(3):679-90.

34. Bascom AT, Sankari A, Badr MS. Spinal cord injury is associated with enhanced peripheral chemoreflex sensitivity. *Physiol Rep*. 2016;4(17):e12948.