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AWARD NUMBER: W81XWH-14-1-0069

TITLE: Advanced Restoration Therapies in Spinal Cord Injury

PRINCIPAL INVESTIGATOR: Visar Belegu, Ph.D.

RECIPIENT: Hugo W. Moser Research Institute at Kennedy Krieger, Inc.
Baltimore, MD 21205

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Baltimore, MD 21205

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14. ABSTRACT

Evidence suggests that functional electrical stimulation (FES) can improve the function of the central nervous system (CNS) after injury or disease. Using FES-based therapies to treat spinal cord injured patients in our clinic, we have observed neurological and physical improvements. Investigations in our basic science research laboratory address the mechanisms by which FES promotes the cellular and molecular CNS regeneration that forms the foundations of this recovery..

SPECIFIC AIM 1: Determine if functional electrical stimulation (FES) in a mouse model of chronic spinal cord injury (SCI) induces proliferation and differentiation of genetically labeled oligodendrocyte progenitor cells (OPCs) (Months 1-12).

SPECIFIC AIM 2: Determine if FES induces remyelination by mature oligodendrocytes in a mouse model of chronic SCI (Months 1-24).

SPECIFIC AIM 3: Determine if functional electrical stimulation in a mouse model of chronic SCI induces cortical plasticity as measured by resting state functional magnetic resonance imaging (rs-fMRI) (Months 1-24).

Relevance: The studies proposed will continue to investigate the role of FES-based restorative therapies in promoting neurological and functional recovery in chronic SCI. We will use existing transgenic mouse lines that enable the genetically labeling of cellular populations to further our understanding of the mechanisms through which FES induces functional recovery. Additionally, we will use a newly developed transgenic mouse lines that enables us to examine the dynamics of myelin formation. We will also further our imaging work by developing methodology to use rs-fMRI for examination of cortical plasticity in response to FES.

15. SUBJECT TERMS					
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SECTION 1 – INTRODUCTION

A spinal cord injury (SCI) disrupts conduction of sensory and motor signals (Belegu et al., 2007). The severity of the injury, and the extent of the neurological impairment following SCI, limits the subsequent neurological recovery. Persons with severe SCI experience limited neurological recovery (Kirshblum et al., 2004; McDonald et al., 2002), and the chances of neurological recovery become even smaller in the chronic phase of SCI (McDonald et al., 2002). Previous studies, however, suggest that through continued rehabilitation efforts, recovery of sensation, function, mobility, and independence in individuals with chronic SCI is possible, months and even years after injury (Harkema et al., 2012; McDonald et al., 2002; Sadowsky et al., 2013). This information has led to increased focus in the development of rehabilitation programs appropriate for individuals with chronic SCI. In particular, activity-based rehabilitation therapy, which aims to induce neurological improvements through continued physical movements, is receiving renewed attention, as studies show that it can effectively increase muscle mass and strength, as well as the independence in activities of daily living (ADL) in individuals with SCI (Harkema et al., 2012; Karimi, 2013; Lorenz et al., 2012; Sadowsky et al., 2013). One of the most widely adapted intervention components of activity-based rehabilitation therapy includes functional electric stimulation (FES) (Harkema et al., 2012; Karimi, 2013; Lorenz et al., 2012). The mechanisms through which FES induces functional recovery remain poorly understood. Remyelination has been suggested as a possible mechanism considering it is an activity-dependent process, however, clinical studies are not sufficient to address this question. Therefore, we have undertaken a preclinical study where we have developed a mouse model of FES in chronically injured mice. In addition, we are using this methodology in transgenic mice that allow us to lineage trace neural progenitor cells that have shown to be induced to proliferate and differentiate upon FES. Furthermore, the current study is allowing us to generate a transgenic mouse that promises to be transformative not only for the study of FES in myelination but in all myelin related pathologies including but not limited to traumatic brain injury, Alzheimer's disease, cerebrovascular insults, and leukodystrophy.

SECTION 2 – KEYWORDS

Spinal cord injury (SCI)

Neural progenitor cells (NPCs)

Functional electrical stimulation (FES)

Neurological recovery

Myelination

Remyelination

SECTION 3 – ACCOMPLISHMENTS

What were the major goals of the project?

SPECIFIC AIM 1: Determine if functional electrical stimulation (FES) in a mouse model of chronic spinal cord injury (SCI) induces proliferation and differentiation of genetically labeled oligodendrocyte progenitor cells (OPCs) (Months 1-12).

SPECIFIC AIM 2: Determine if FES induces remyelination by mature oligodendrocytes in a mouse model of chronic SCI (Months 1-24).

SPECIFIC AIM 3: Determine if functional electrical stimulation in a mouse model of chronic SCI induces cortical plasticity as measured by resting state functional magnetic resonance imaging (rs-fMRI) (Months 1-24).

What was accomplished under these goals?

SPECIFIC AIM 1

1) Major activities: We purchased and bred nestin-CreER, Z/EG and ROSA-YFP mice. Performed experiments to determine which reporter transgenic line labels sufficient and specific NPCs in a chronic model of contusive SCI for use in future experiments. Performed experiments to determine the most suitable amount of 4-hydroxytamoxifen to use to label sufficient and specific NPCs in a chronic model of contusive SCI. Performed experiments to determine if BrdU or EdU (and the appropriate concentration and amount) is the most suitable to label sufficient and specifically dividing cells in a chronic model of contusive SCI for use in future experiments. Performed experiments in mice with C57BL6 genetic background to determine if FES enhances cell proliferation in mice that are in chronic stages of contusive SCI. Performed experiments in mice with C57BL6 genetic background to determine their neurological recovery in response to FES in a chronic model of contusive SCI.
2) Specific objectives: Determine which reporter transgenic line labels sufficient and specific NPCs in a chronic model of contusive SCI. Determine the most suitable amount of 4-hydroxytamoxifen to use to label sufficient and specific NPCs in a chronic model of contusive SCI. Determine if BrdU or EdU (and the appropriate concentration and amount) is the most suitable to label sufficient and specifically dividing cells in a chronic model of contusive SCI for use in future experiments. Determine if FES enhances cell proliferation in mice that are in chronic stages of contusive SCI. Determine if FES enhances neurological recovery in mice that are in chronic stages of contusive SCI.
3) Significant results or key outcomes: We determined that ROSA-YFP mice reporter line labels sufficient and specific NPCs in a chronic model of contusive SCI; we will use nestin-CreER+/-;ROSA-YFP+/- in our future experiments. We determined that 5 mg of 4-hydroxytamoxifen administered over a 5-day period is to label sufficient and specific NPCs in a chronic model of contusive SCI; we will use this amount of 4-hydroxytamoxifen in our future experiments. We determined that 25 mg/kg of EdU is the most suitable method to label sufficient and specifical dividing cells in a chronic model of contusive SCI; we will use this concentration of EdU in our future experiments. Completed the experiment designed to determine if FES enhances neurological recovery in mice that are in chronic stages of contusive SCI. We are currently analyzing the data from the neurological assays performed during this experiment; additionally, we are processing the tissue from this experiment for anatomical analysis. Furthermore, we are considering methods to improve the method of delivering FES. We have injured 20

C57BL6 genetic background with 50 kdyn SCI. In these mice, we will soon implant electrodes in order to administer FES so we can determine if FES enhances cell proliferation in mice that are in chronic stages of contusive SCI; we will apply the lessons learned from the previous experiment with regard to the best methods of delivering FES.

4) Other achievements: N/A

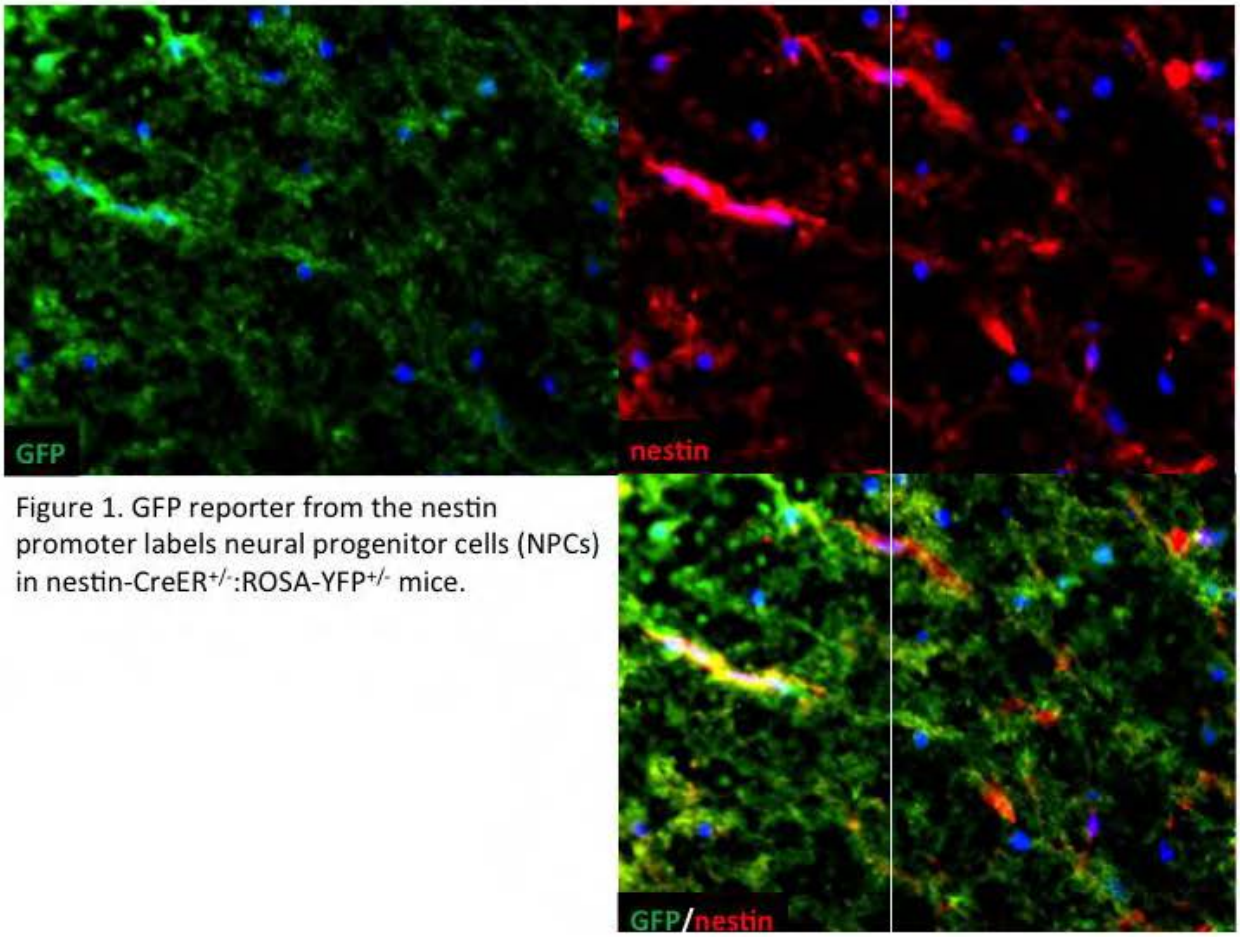
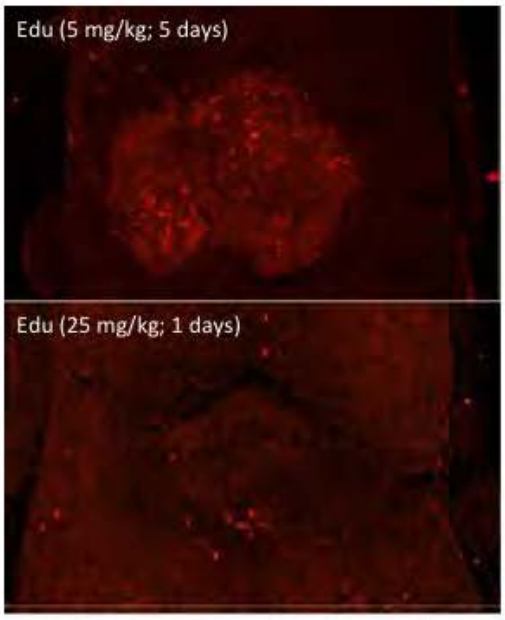


Figure 1. GFP reporter from the nestin promoter labels neural progenitor cells (NPCs) in nestin-CreER$^{+/-}$:ROSA-YFP$^{+/-}$ mice.



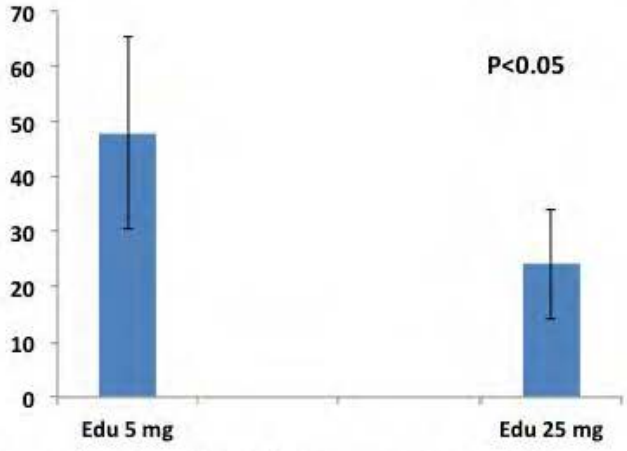


Figure 2. EdU administration in chronically injured mice. While Edu administered over 5 days in 5 mg/kg concentration labels more dividing cells, these cells are predominantly in injury epicenter (non-neural lineage).

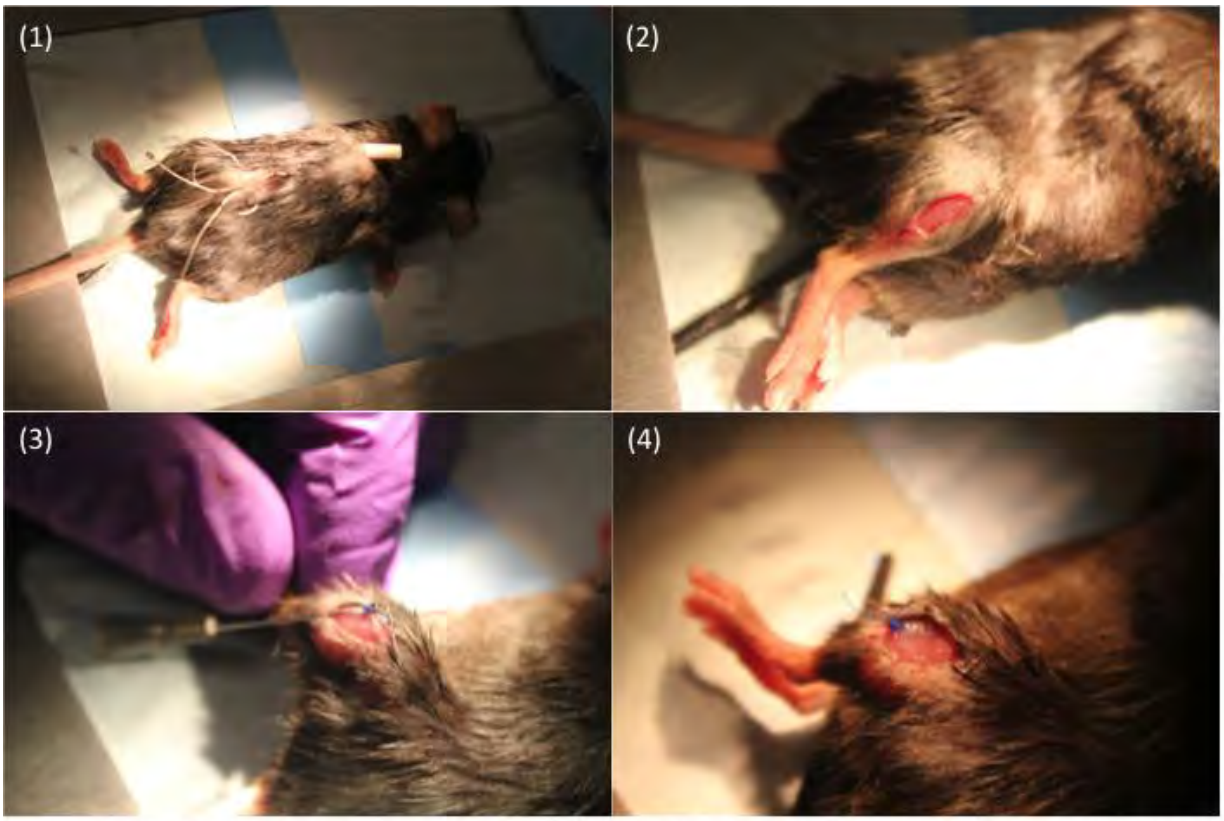


Figure 3. Electrode implantation in the mice. (A) Electrodes are shuttled underneath the skin, down to the lower back. (B) An incision is made near the common peroneal nerve, (C) the electrode is implanted (without touching the nerve), and is sutured to the muscle.

SPECIFIC AIM 2

1) Major activities: We designed and generated mice with a transgene we have termed MBP_MBP-CTRN_MBP-RFP. Breed the founders with C57BL6 genetic background in order to backcross into this genetic background and create a congenic line. Breed the heterozygous MBP_MBP-CTRN_MBP-RFP with B6.129S4-Gt(ROSA)26Sortm2(FLP*)Sor/J (FLP*) mice which are also in a C57BL6 genetic background. Breeding heterozygous MBP_MBP-CTRN_MBP-RFP mice. Examined expression of CTRN transgene and the endogenous MBP gene in the heterozygous MBP_MBP-CTRN_MBP-RFP mice. Completing electron microscopy analysis of the structure of the myelin sheets in the spinal cord and brain of the heterozygous MBP_MBP-CTRN_MBP-RFP. Developed a qPCR genotyping assays for the following transgenic elements in MBP_MBP-CTRN_MBP-RFP mice: CTRN, RFP, neo cassette, flipase, Cre, and endogenous MBP.
2) Specific objectives: Generate a congenic heterozygous MBP_MBP-CTRN_MBP-RFP transgenic line. Confirm that the expression of the transgene parallels expression of endogenous MBP.

3) Significant results or key outcomes: We have obtained MBP_MBP-CTRN_MBP-RFP transgenic mice with and without the neo-cassette. We have confirmed that the expression of the transgene matches expression of endogenous MBP in the spinal cord and brain of the transgenic mice. We are currently confirming the structure of the myelin sheets in these transgenic mice.
4) Other achievements: N/A

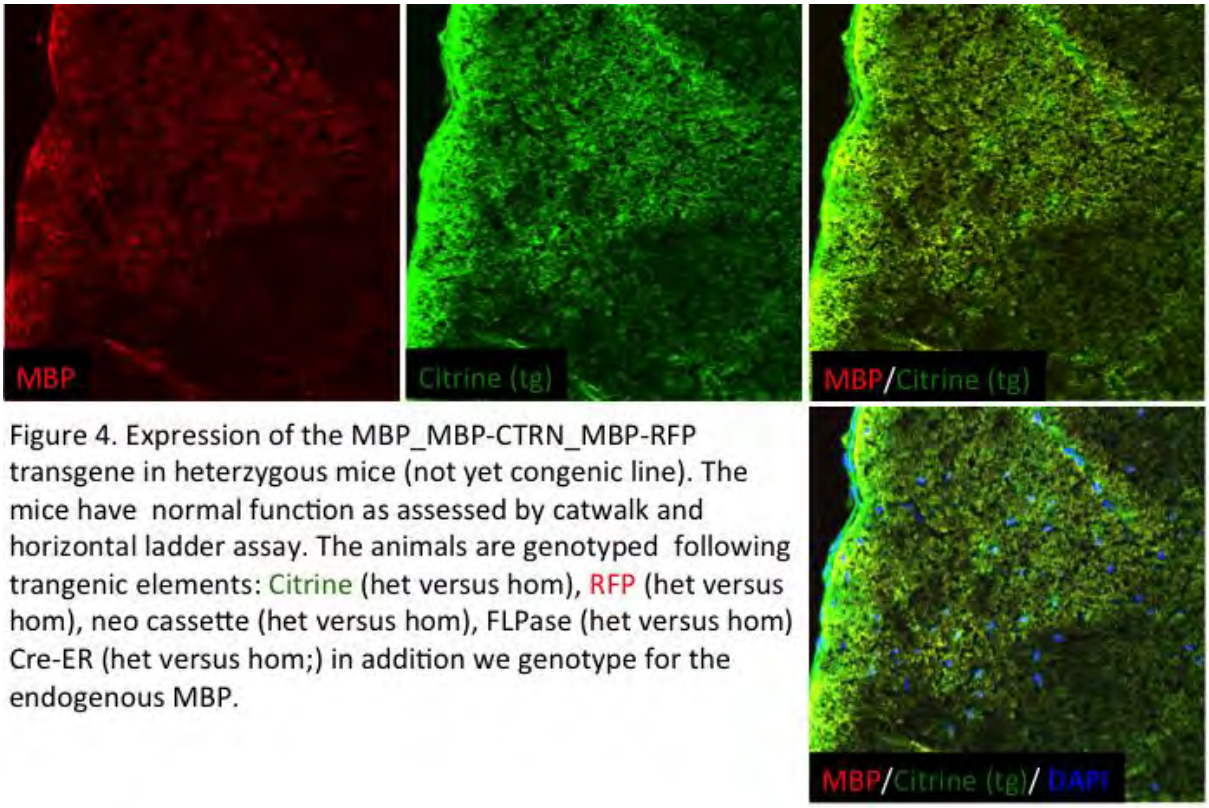


Figure 4. Expression of the MBP_MBP-CTRN_MBP-RFP transgene in heterzygous mice (not yet congenic line). The mice have normal function as assessed by catwalk and horizontal ladder assay. The animals are genotyped following trangenic elements: Citrine (het versus hom), RFP (het versus hom), neo cassette (het versus hom), FLPase (het versus hom) Cre-ER (het versus hom;) in addition we genotype for the endogenous MBP.

SPECIFIC AIM 3

1) Major activities: We are currently awaiting approval from the F.M. Kirby Research Center High-Field Preclinical MR Facility.
2) Specific objectives: Obtain approval for and image mice in a chronic model of contusive SCI using a resting-state magnetic resonance imaging acquisition sequence. Perform spinal cord injuries in mice that will undergo resting state fMRI.
3) Significant results or key outcomes: We have performed spinal cord injuries in three adult (12 week old) mice.
4) Other achievements: The committee at F.M. Kirby Research Center High-Field Preclinical MR Facility has approved our proposed acquisition sequence (a gradient echo EPI sequence) and the data analysis methods (whole brain seed correlation analysis and interhemispheric FC was evaluated with a pairwise seed analysis). We have been advised

to change our proposed anesthesia method from isofluorane to medetomidine. We have made the appropriate changes and are awaiting final approval.

What opportunities for training and professional development has the project provided?

The current project enabled the PI to organize a training course for SCI in collaboration with Dr. Aileen Anderson from the Christopher and Dana Reeve Foundation. During this training course the laboratory staff at the International Center for Spinal Cord Injury learned to perform (1) laminectomies in mice and (2) contusion spinal cord injuries. In addition, following that training course, the PI continued staff training in surgical procedures to implant electrodes for electrical stimulation in mice.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

SPECIFIC AIM 1:

1) Evaluate the neurological recovery in mice that were treated with FES in chronic stages of contusive SCI.
2) Complete and evaluate the cellular proliferation in mice treated with FES in chronic stages of contusive SCI.
3) Depending on these results we will breed nestin-CreER:ROSA-YFP mice for experiments to investigate the involvement of NPCs in neurological recovery in mice treated with FES in chronic stages of contusive SCI.

SPECIFIC AIM 2:

1) Evaluate the structure of myelin sheaths in MBP_MBP-CTRN_MBP-RFP transgenic mice.
2) Generate homozygous MBP_MBP-CTRN_MBP-RFP transgenic mice and continue to derive congenic MBP_MBP-CTRN_MBP-RFP transgenic mice.

SPECIFIC AIM 3:

1) Scan and process resting state MRI data with control and injured animals as soon as we receive approval from the committee at F.M. Kirby Research Center High-Field Preclinical MR Facility.

SECTION 4 - IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

SECTION 5 - CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

We originally anticipated purchasing a Neurolucida and AutoNeuron Neuron Tracing System in Year 1 to analyze data obtained in Aims 1 & 2, we now plan to complete that purchase early in Year 2 instead.

Additionally, a self-funded post-doctoral fellow worked on this project for Year 1, this lowered our salary expenditures. He has recently resigned and will need to be replaced in Year 2 with another post-doctoral fellow or laboratory technician. Therefore, we anticipate that our personnel expenditures will increase in Year 2 as a result.

In Year 1, we spent less than anticipated on reagents such as 4-hydroxytamoxifen and EdU because we performed experiments with C57B16 mice. These expenses will increase in Year 2.

Additionally, we have begun using the Johns Hopkins Microscope Facility but due to a delay in the billing, we have not incurred those expenses yet but they are pending.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

N/A

SECTION 6 – PRODUCTS

Publications, conference papers, and presentations

Nothing to Report.

Journal publications

None

Books or other non-periodical, one-time publications.

None

Other publications, conference papers, and presentations.

None

Website(s) or other Internet site(s)

None

Technologies or techniques

none

Inventions, patent applications, and/or licenses

None

Other Products

To date we have generated several transgenic mouse lines that promise to be transformative in the study of oligodendrocytes and myelination during development and in various pathologies that are pertinent to the healthcare of military personnel.

SECTION 7 - PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Visar Belegu, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9.6 months
Contribution to Project:	Dr. Belegu has supervised all the work performed in this project. In addition, Dr. Belegu has performed SCI surgeries, electrode implantations, FES stimulation, and neurological assays assisted by Dr. Liu and Ms. Miglioretti.

Name:	Ali Fatemi, MD
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	4.8
Contribution to Project:	Dr. Fatemi has overseen and analyzed the data obtained in Specific Aim 1 and 2.

Name:	Michael Johnston, MD
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3.6
Contribution to Project:	Dr. Johnston has overseen and analyzed the data obtained in Specific Aim 1 and 2. Dr. Johnston has advised Dr.

	Belegu on future directions with regard to experimental design within this project.
Name:	Su Liu, MD
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	11.4
Contribution to Project:	Dr. Liu has assisted Dr. Belegu in performing SCI surgeries electrode implantations, and FES stimulation. In addition, she has performed the perfusions, staining and imaging for animals in Specific Aim 1 and 2.
Name:	Anna Miglioretti, BS
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	Mrs. Miglioretti has performed all the genotyping for animals in Specific Aim 1 and 2. She has assisted in SCI surgeries, FES stimulation, and neurological assays. Additionally, she has assisted in dissecting brains and spinal cords from animals for Specific Aim 1 and 2.
Name:	Pradeep Manoharan, MD
Project Role:	Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9
Contribution to Project:	He has assisted with neurological assays and animal care. Additionally, he has assisted in dissecting brains and spinal cords from animals for Specific Aim 1 and 2.
Name:	John McDonald, MD, PhD
Project Role:	Consultant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	.02
Contribution to Project:	Dr. McDonald consulted with Dr. Belegu on expression of MBP-CTRN in the spinal cord and 4-hydroxytamoxifen and EdU administration.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Organization Name: N/A

Location of Organization: N/A

Partner's contribution to the project: N/A

Financial support: N/A

In-kind support: N/A

Facilities: N/A

Collaboration: N/A

Personnel exchanges: N/A

Other: N/A

SECTION 8: SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: Next Page

Advanced Restoration Therapies in Spinal Cord Injury

13211006
W81XWH-14-1-0069

PI: Visar Belegu, PhD **Org:** Hugo W. Moser Research Institute at Kennedy Krieger, Inc.

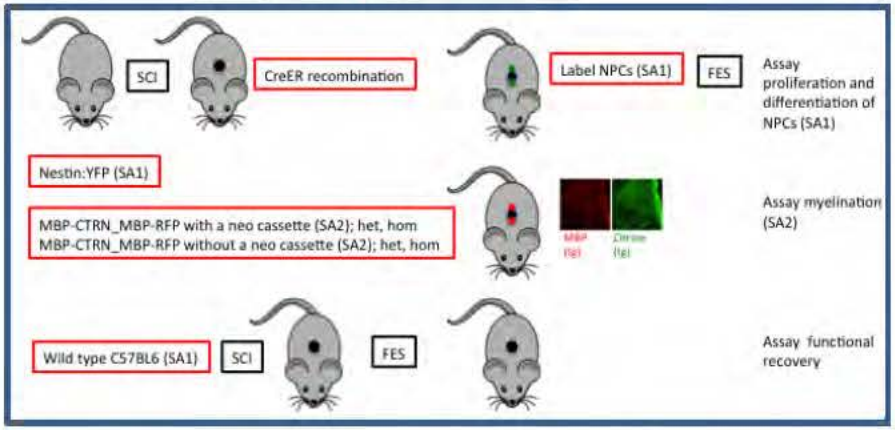
Award Amount: $1,032,569.11
(+ $965,926 – year 2)

Study/Product Aim(s)

- SPECIFIC AIM 1: Determine if functional electrical stimulation (FES) in a mouse model of chronic spinal cord injury (SCI) induces proliferation and differentiation of genetically labeled oligodendrocyte progenitor cells (OPCs)
- SPECIFIC AIM 2: Determine if FES induces remyelination by mature oligodendrocytes in a mouse model of chronic SCI
- SPECIFIC AIM 3: Determine if FES in a mouse model of chronic SCI induces cortical plasticity as measured by resting state functional magnetic resonance imaging (rs-fMRI)

Approach

We aim to investigate the role of functional electrical stimulation in generating neurological recovery following spinal cord injury. Specifically, we will use transgenic mice to investigate if such an effect is mediated through cells of the oligodendrocyte lineage.



Accomplishments: (1) ROSA-YFP is appropriate reporter line for future experiments. (2) determined optimal dose of 4-hydroxytamoxifen for labelign NPCs. (3) determine optimal does EdU (not BrdU) to label dividing cells. Completed an experiment to determine if FES enhances neurological recovery in mice that are in chronic stages of contusive SCI. Confirmed that the transgene in SA2 parallels MBP expression.

Timeline and Cost

Activities	Year 1	2
Determine if FES induces proliferation & differentiation of OPCs		
Determine if FES induces remyelination by mature oligodentrocytes		
Determine if FES induces cortical plasticity		
Estimated Budget ($K)	**$1,033**	**$966**

Goals/Milestones (Example)

Y1 Goal – System demonstration

- ☑ Obtain regulatory approval for animal work from Johns Hopkins IACUC and USAMRMC ACURO.
- ☑ Initiate generation of transgenic animals.
- ☑ Initiate and completed SCI, and FES implantation, and electrical stimulation for two sets of experiments.
- ☐ Develop fMRI sequences and data processing pipeline.

Y2 Goals – System validation

- ☐ Complete the analysis on the effects of FES in proliferation and differentiation of spinal cord progenitor stem cells.
- ☐ Complete the analysis on the effects of FES in myelination.
- ☐ Complete the analysis on the effects of FES in neurological recovery.

Comments/Challenges/Issues/Concerns

- The final goal of Year One has been delayed due to approvals from the FM Kirby Research Center for our protocol. We have made the appropriate changes and are awaiting final approval.
- We are underspent for Year One due to delaying the equipment purchase and use of a self-funded post-doctoral fellow. The equipment will be purchased in Year Two. The self-funded fellow recently resigned and in Year Two we will need to hire either a funded post-doctoral fellow or laboratory technician to fill that position.

Budget Expenditure to Date

Projected Expenditure: $1,032,569.11
Actual Expenditure: $634,547.97 (through March 2015)

Updated: 05/11/2015

SECTION 9: APPENDICES

REFERENCES FOR INTRODUCTION:

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