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TITLE: Recombinant GABAergic cells as a therapy for chronic neuropathic pain

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: The main focus of the project is a development of recombinant cell-based therapy for chronic pain. Scope: The reduction in the GABA signaling and its relation to the development of chronic pain has been described after spinal cord and peripheral nerve injuries. Transplantation of GABAergic neuronal cells may restore the inhibitory potential in the spinal cord and replace dysfunctional interneurons. Grafted cells may also release additional analgesic peptides by means of genetic engineering to further enhance the benefits of this approach. Conopeptides are ideal candidates for recombinant expression using cell based strategies. The goal of the project is to develop transplantable recombinant GABAergic cells releasing MVIIA that can alleviate pain-like behavior in models of neuropathic pain after peripheral and spinal cord injury. Major findings: We have engineered and characterized the GABAergic progenitors expressing MVIIA. Recombinant and nonrecombinant cells were intraspinally injected into animals in the models of peripheral nerve injury and spinal cord injury. We have observed beneficial effects of the grafted cells in reducing hypersensitivity in all grafted animals, especially in the recombinant group. Injection of MVIIA antibody reduces the analgesic effect of the recombinant graft. The level of pain-related cytokines was reduced in the grafted animals and correlation between these pain markers and actual behavior was detected.

15. SUBJECT TERMS
neuropathic pain, nerve injury, spinal cord injury, cell therapy, GABAergic cells, conopeptides, MVIIA, animal models

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1. INTRODUCTION

Chronic pain is a clinically challenging condition often associated with the development of tolerance and addiction to analgesic drugs. Targeted therapy might overcome these issues and improve the management of chronic pain. One the key events underlying development of chronic pain is reduced inhibition in the spinal cord, causing misinterpretation of the incoming signal from the periphery. Dysfunctional signaling of GABA as an inhibitory neurotransmitter is suggested as the major cause of neuronal hyperexcitability. Pharmacological targeting of GABA receptors is insufficient to rebalance the spinal signaling due to widespread location of GABAergic receptors throughout the CNS. Transplantation of GABAergic cells showed reduction of chronic pain and partial restoring of the inhibitory balance in the spinal cord. To improve the analgesic outcome of this approach, cells may be engineered to produce additional analgesic peptides. The benefits of using recombinant cells are that it allows targeting multiple pain-processing pathways, to rebalance inhibitory signaling and to replace dysfunctional neurons at the same time. In this proposal, as a recombinant peptide produced by GABAergic cells, conotoxin MVIIA is investigated in animal models of peripheral and central neuropathic pain. Conotoxin MVIIA is an FDA approved therapeutic peptide for the treatment of chronic neuropathic pain. However, due to its poor penetration through blood brain barrier it must be delivered via intrathecal catheters. MVIIA produced by grafted cells might provide more targeted pain control and improve the quality of life of affected patients.

2. KEYWORDS

neuropathic pain, nerve injury, spinal cord injury, cell therapy, GABAergic cells, conopeptides, MVIIA, animal models

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major Task 1: IACUC and ACURO approvals
Completion: 100%

Major Task 2: Engineering of recombinant cells
Completion: 100%

Major Task 3: Induction of peripheral and central chronic pain
Completion: 100%

Major Task 4: Histochemical and biochemical evaluation of the therapy
Completion: 80%

What was accomplished under these goals?

Major Task 1: IACUC and ACURO approvals

As stated in the previous report cycle, all necessary approvals have been obtained, project workflow was designed, and personnel was recruited.

Major Task 2: Engineering of recombinant cells

Major activities:
Subtask 1: Personnel training for cell culture methods
Subtask 2: Harvesting E14 cell, culturing, transformation with lenti-MVIIA

Subtask 3: Evaluation of recombinant cell survival and phenotype
Subtask 4: Quantification of MVIIA production by recombinant cells and optimization of culture environment

Specific objectives:

1) Training of the new personnel, including safety rules and general lab methods, together with specific training and IACUC approval on animal protocols are prerequisite for participation on the research projects.
2) Cells from medial ganglionic eminence at E14 stage of rat embryos are harvested to obtain a population rich in GABAergic cells. Cells are transfected with lentiviral vector encoding MVIIA to engineer recombinant cells.
3) To evaluate cell survival and possible phenotypic changes induced by transfection or culture environment.
4) To evaluate the stability and proper folding of recombinant MVIIA peptide and abilities of cells to produce and release MVIIA.

Results:
1) As stated in the previous report cycle, Ms. Hernandez has been trained for all necessary procedures involving cells culture, animal surgeries and behavioral testing and lab techniques.
2) Cells have been harvested and transduced for each cohort of animals; only fresh cells were used for all transplantation. Cells were not kept frozen due to low survival rate observed in our previous experiments after thawing. E14.5 fetal neocortical tissue from Sprague-Dawley rats was microdissected into Hank's balanced salt solution and a cell suspension created via mechanical trituration. Cells were plated at an initial concentration of 5x105cells/ml of the culture media containing 10ng/ml of human recombinant basic fibroblast growth factor (FGF-2; Sigma) in 75 cm2 treated cell culture flasks (Corning). 24 hours post-harvest cells were transduced with lentivector encoding MVIIA at $1x10^{11}$ viral particles/ml for 4 hours. Media was changed and cell were replated into $70cm^2$ culture flasks.
3) Cell survival has been evaluated before each transplantation. The culture conditions were optimal to keep survival rate around 80% as detected by Trypan blue test. From each cohort of cells used for transplantation, a small portion was used for immunostaining to confirm the phenotype of the cells. Cells were cultured for 3-4 days and their viability was estimated using Trypan blue solution and hemocytometer. The average viability at 3-4 days post lentiviral transduction was 78.3% which is suitable for grafting procedures. Cells were then plated into 12 well plates or 8 well chambers coated with poly-L-ornithine/fibronectin at concentration 5x105/well and incubated at 37°C for 2-3 days. Cells were fixed with 4% paraformaldehyde, washed and incubated in 5% normal goat serum for 2 hours and overnight in primary antibodies (GABA, 1:200, Sigma; βTubulin 1:1000, Sigma; GFAP, 1:1000, Sigma; MVIIA, 1:50, 21st Century Biochemicals) followed by incubation with appropriate secondary antibodies (Alexa Fluor 488, 594, anti-rabbit, anti-mouse, 1:250, Invitrogen). After final wash the upper structure of the chamber was carefully removed and cells were coverslipped (VectaShield, Vector).

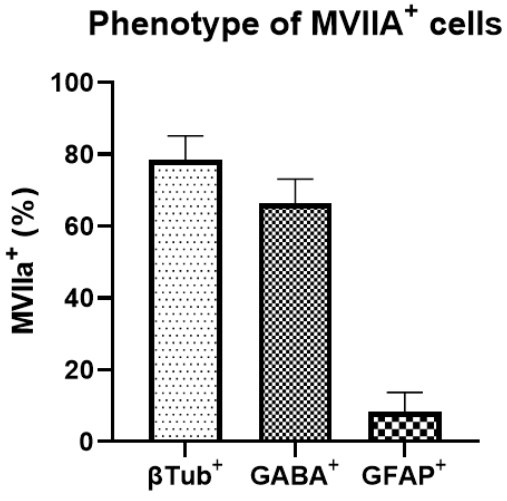

Fig. 1: Phenotype of MVIIA+ grafted cells.

The transduction efficiency was estimated based on number of MVIIA+ cells out of β tubulin+ cells. Using β tubulin positive cells as a marker can assure those cells are accessible to the staining and provide better estimate of the transduction rate. β tubulin was also selected based on the observation that MVIIA+ signal was almost exclusively detected within the cells that express β tubulin.
In the previous period transduction efficiency was estimated at 83.6% of β tubulin+ cells. We have further quantified the phenotype of MVIIA positive cells by double labeling with βTubulin, GABA and GFAP antibodies. The majority of MVIIA signal was detected in βTubulin (78.3%) or GABA (66.3%) positive cells, indicating neuronal phenotype. Colocalization with astroglial marker GFAP was detected in 8.5% of MVIIA positive cells (Fig. 1).

4) Analysis of cell supernatant for the presence of MVIIA protein has been conducted during the previous period. In the current experiment, the effect of MVIIA actively released from the cells has been evaluated in vivo by intrathecal injection of MVIIA antibody, followed by behavioral testing of animals. Results are in the section Major Task 3.

Major Task 3: Induction of peripheral and central chronic pain

Major activities:
Subtask 1: Surgeries for chronic constriction injury (CCI) model, train personnel for behavioral testing and animal handling
Subtask 2: Transplantation of recombinant and nonrecombinant cells, saline injections
Subtask 3: Behavioral evaluation of the treatment in CCI model
Subtask 4: Surgeries for spinal cord injury (SCI) model
Subtask 5: Transplantation of recombinant and nonrecombinant cells, saline injections
Subtask 6: Behavioral evaluation of the treatment in SCI model

Specific objectives:
1) To induce chronic pain after peripheral nerve injury using specific animal model and ensure personnel ability to follow the procedures.
2) As a proposed therapy to alleviate chronic pain, recombinant cells are grafted into the spinal cord in animal models. Non-recombinant cells and saline injections serve as controls.
3) Evaluation of the analgesic effect of the grafted cells in the CCI model
4) To induce chronic pain after spinal cord injury for the evaluation of cell therapy.
5) To develop the model for evaluation of analgesic propertied of grafted cells in SCI animals.
6) Evaluation of the analgesic effect of the grafted cells in the SCI model.

Results:
1-3) Objectives listed as 1-3 have been addressed in the previous report cycle. In this period the main focus was on induction of SCI model and on histochemical and biochemical analysis of the tissue.

4) Male and female Sprague Dawley rats (Envigo, IN) underwent surgery for spinal cord injury. Rats were anesthetized with 4-5% isoflurane in O_2 and maintained on 2-3% isoflurane/O_2. 2-3 thoracic vertebrae were exposed, and a laminectomy was performed to exposed spinal cord segments T6-T8. An aneurism clip 1 mm wide (20 g compression force; Harvard Apparatus) was oriented in the vertical position and a spinal segment in the area between T6- T7 was compressed for 60 sec. The clip was then removed, and the wounds closed. Following spinal compression, the bladder was expressed twice daily for 7-10 days, or until voiding was regained. Animals with grafted cells received daily cyclosporine injections starting 2 days prior the surgery.

5) For transplantation, cells were pelleted (1500 rpm/3 min) and resuspended in Hanks media at a concentration of 50,000 cells/µl. Cells were transplanted at 5 weeks post SCI to target chronic stages of pain development in this particular model. Cells were injected bilaterally in the superficial dorsal horn at L3-L5 with 10 µl Hamilton syringe attached to a pulled glass pipet (diameter ~50 µm). A small puncture was made in the meninges and 1.0 µl of cell suspension (~5 x104 cells) was stereotactically injected. Following transplantation, the area was covered with elastic sheathing, the overlying musculature sutured, and the skin closed with clips. Control animals received saline injection. Animals with grafted cells received daily cyclosporine injections starting 2 days prior to the surgery.

6) Behavior
Animals were tested for the presence of tactile, cold and heat hyperalgesia using standard pain tests. Locomotor scores were assessed using Basso-Beattie- Bresnahan test. Place escape avoidance test was used to further evaluate the presence of ongoing pain.

Tactile hypersensitivity: The threshold level to an innocuous mechanical stimulus was measured with calibrated von Frey hairs ranging from 0.4 to 15 g. Animals were placed beneath an inverted clear plastic cage on an elevated wire mesh floor. Calibrated von Frey filaments were applied to the plantar skin of the hind paw

with increasing force. The withdrawal threshold was taken as the lowest force (g) that evokes a brisk hind paw withdrawal response, with vocalization, head turns towards stimulus.

Cold hypersensitivity: Sensitivity to a non-noxious cooling stimulus was evaluated using acetone. 100 µl of acetone was dropped onto the lateral margin on the hind paw from a blunted 22 ga needle attached to a syringe. Acetone was applied to the hind paw 5 times, with about 1-2 min between applications. The total number of positive responses out of five were converted to a percent response frequency.

Heat hypersensitivity: Rats were placed beneath an inverted clear plastic cage on an elevated glass floor and a radiant heat source beneath the glass was aimed at the plantar hind paw which activates a timer.) Withdrawal latencies are the length of time between the activation of the heat source and the hind paw withdrawal from the glass (normal baseline ~10 sec). To avoid tissue damage in the absence of a withdrawal, the cutoff was set at 20 sec. The average latency was calculated from 3 trials with 30 sec apart.

Open field test: The Basso-Beattie- Bresnahan (BBB) test was used for evaluation of motor behavior. Rats were placed in the center of an open-field area with a 4-foot diameter, and the behavior of the animals is

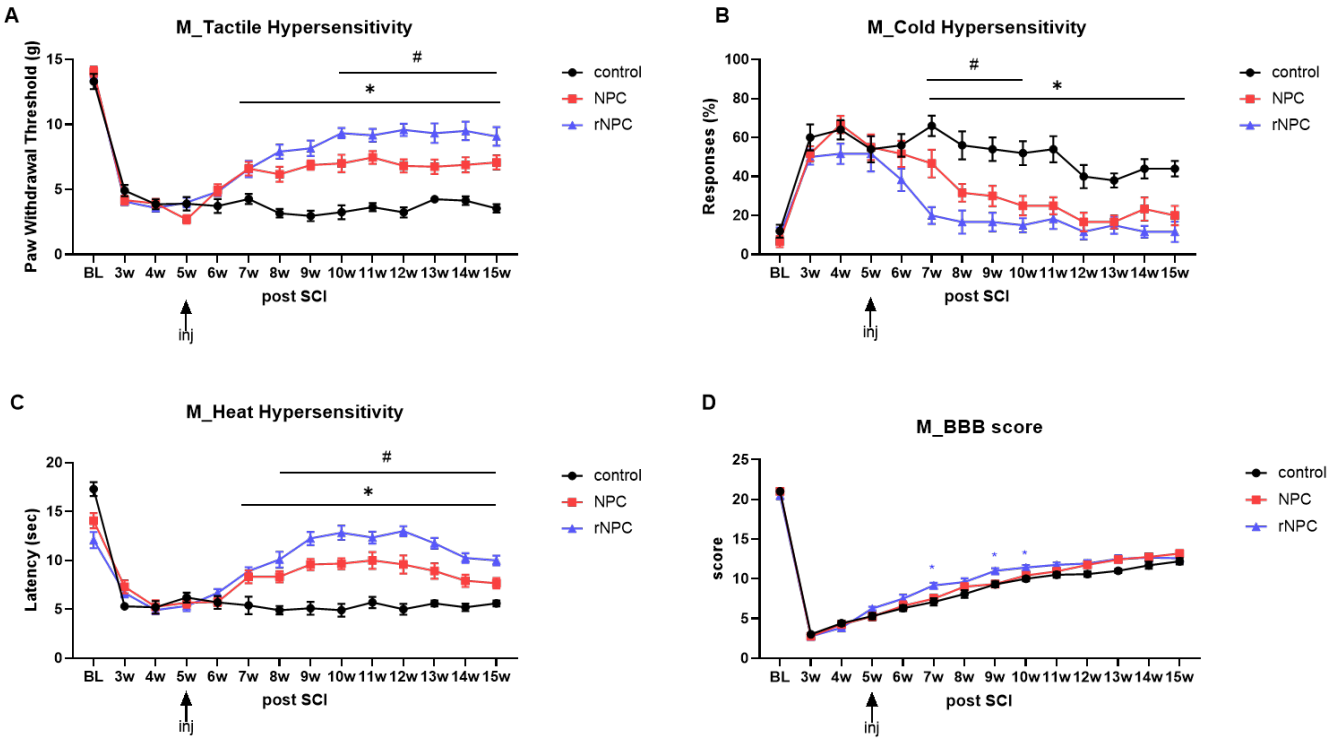

Fig. 2: Behavioral evaluation of A) tactile B) cold C) heat hypersensitivity and D) locomotor score in SCI male rats with different treatment. *p<0.05 vs control, #p<0.05 between grafted animals

observed for a 4 min test period by two individuals blinded to the treatment. The scale was designed to reflect motor rating scores, ranking from zero which indicates complete paralysis without joint movement to 21 which indicates normal locomotion with full coordination and proper gait, movement at all joints, full weight support, and appropriate limb, body and tail positioning. BBB score was not affected by the treatment, as expected.

Place escape avoidance: To evaluate ongoing pain incorporating cognitive and motivational aspects, a place escape avoidance test box was used. Animals are placed in the middle of a 2-chambered box to habituate and then stimulated with von Frey filaments upon entry into the usually preferred dark side but not in the light side. The amount of time spent in the light side is measured. Significant increase in the time spend at the dark side was considered as a positive outcome of the therapy.

Males (Fig. 2): Spinal cord injury induced hypersensitivity to tactile (A), cold (B) and heat (C) stimuli, was observed by week 3 post injury. Spinal injection of NPC and rNPC progressively reduced hypersensitivity compared to control animals with saline injection. The difference was significant starting 2 weeks post injection (7w post injury) till the end of experiment ($p<0.05$). Differences between the groups with NPC and rNPC were observed between 5-10 weeks post injection for tactile (A, $p<0.05$), 2-5weeks post injection for cold (B, $p<0.05$) and 3-10 weeks post injection for heat (C, $p<0.05$) hypersensitivity. Locomotor score using BBB scale was not

significantly different overall, but there were subtle changes between animals with rNPC and control group, between 3-5 weeks post injection.

Females (Fig. 3): Behavioral response of female rats was similar to males in general, although there were differences in the effect of the nonrecombinant and recombinant cells. All animals developed hypersensitivity to tactile and thermal stimuli post injury. Hypersensitivity remains present in control animals, although a partial drop in cold hypersensitivity was observed 15 weeks. NPC and rNPC reduced hypersensitivity compared to control animals started at 2 weeks post injection. Differences between NPC and rNPC were observed at weeks 5-7 and 9-10 post injection for tactile (A, $p<0.05$), weeks 6-10 post injection for heat, weeks 3-4 and 6-10 post injection for cold. BBB score was comparable between treatment groups. In general, female rats showed better recovery and less issues with inflammation of the urinary tract than male rats.

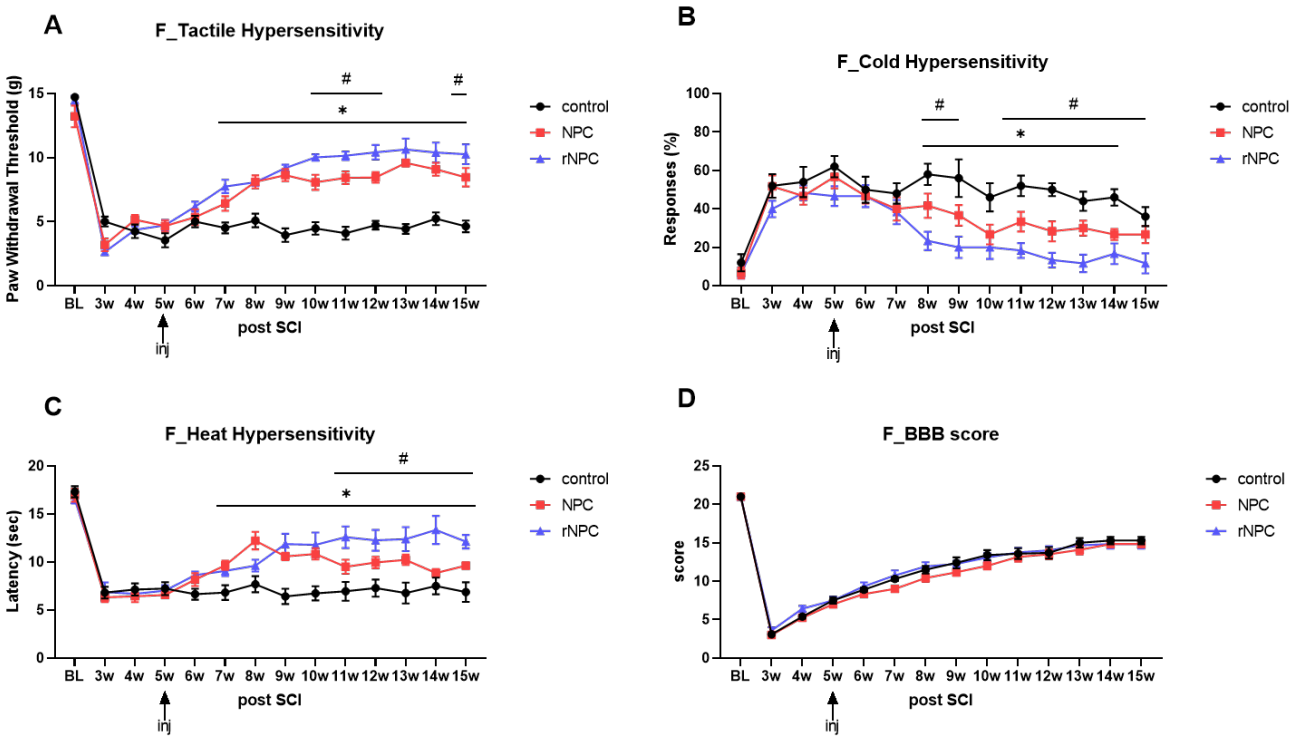

Fig. 3: Behavioral evaluation of A) tactile B) cold C) heat hypersensitivity and D) locomotor score in SCI female rats with different treatment. *p<0.05 vs control, #p<0.05 between grafted animals

To further evaluate the analgesic effect of the treatment, animals were tested using a place escape avoidance method. Both males and females treated with saline developed an avoidance behavior when light tactile stimuli were present in the preferred side of the cage, suggesting the presence of ongoing pain. NPC and rNPC treated animals did not show significant difference (avoidance) in the time spent in the preferred side despite the presence of tactile stimuli (Fig. 4).

The analgesic effect of MVIIA released from the recombinant cells was evaluated in tactile and cold hypersensitivity tests in animals with recombinant graft after intrathecal injection of MVIIA antibody.Rats were anesthetized with 4-5% isoflurane in O_2 and maintained on 2-3% isoflurane in O_2 and placed in a modified stereotaxic frame. The atlanto-occipital membrane was be exposed, sterile intrathecal catheter (ReCathCo, Inc.) was introduced into the intrathecal space, with the tip positioned over the lumbar area. Animals were left to recover for at least 3 days before drug injection. 5ul of drug followed by 5ul of saline was injected on the test day, followed by behavioral testing. On the first day, animals were injected with saline and tested 30 mins post injection. Next day animals were injected with MVIIA antibody and tested. Baseline values were recorded before each injection.
Injection of saline did not induce any significant changes hypersensitivity scores. Injection of MVIIA antibody led to reduction of analgesic effect of the treatment and increase of hypersensitivity in both tests and both

sexes with p<0.05, except for female animals that responded more robustly to MVIIA antibody injection in the cold hypersensitivity test with p<0.001 (Fig. 5).

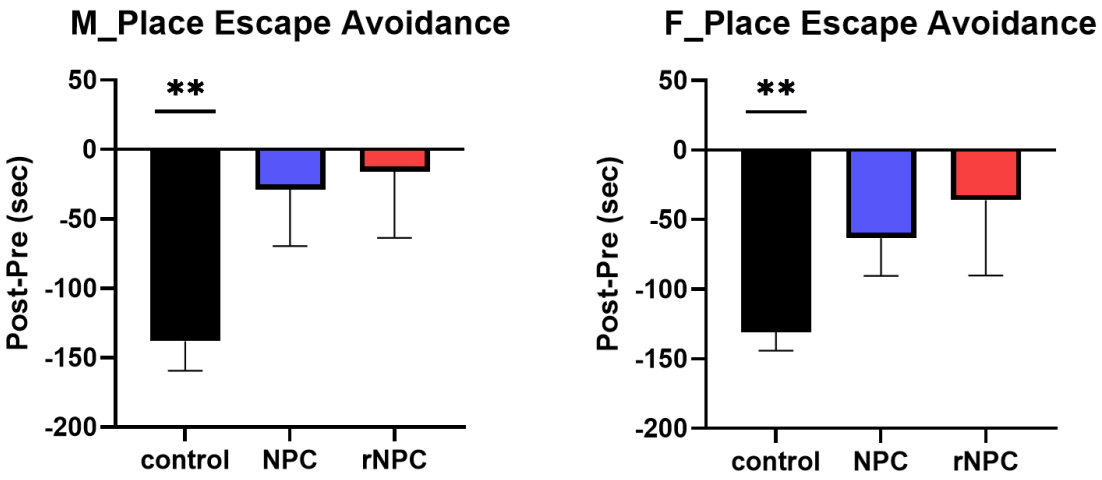

Fig. 4: Place escape avoidance behavior. Control, saline treated animals display avoidance behavior-less time spent on the preferred side of the cage in the presence of a tactile stimuli. **p<0.01 vs null hypothesis

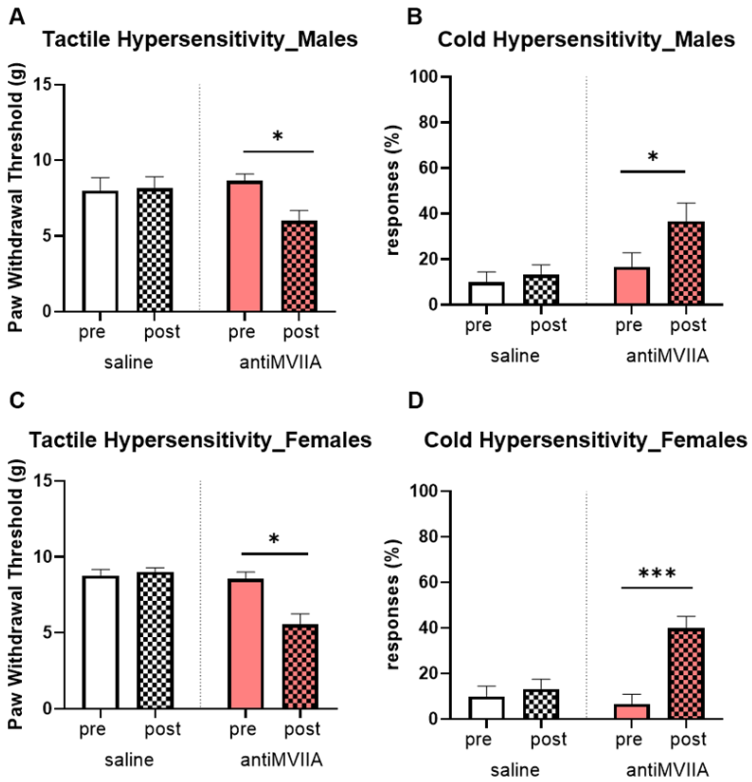

Fig. 5: Intrathecal injection of MVIIA antibody reduced analgesic effect of the recombinant graft evaluated as A,C) tactile and B,D) cold hypersensitivity in male and female animals. *p<0.05, ***p<0.001.

Major Task 4: Histochemical and biochemical evaluation of the therapy

Major activities:
Subtask 1: Perfusion, tissue harvesting and processing for immunostaining
Subtask 2: Biochemical and histochemical evaluations.

Specific objectives:
1) To harvest spinal cord tissue from the experimental animals after behavioral test are finalized and to prepare the tissue for biochemical or histochemical analysis.
2) To evaluate the distribution, survival and the physiological effect of graft in the spinal cord tissue.

Results:
1) All animals have been perfused or sacrificed and tissue was harvested for immunohistochemical and biochemical evaluation. Animals were deeply anesthetized and intracardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords were removed and post-fixed overnight, followed by incubation in 30% sucrose for 48 hours. Serial sections cut on cryostat at 40 μm are collected either as slide mounted or free floating. For biochemical analysis, tissue samples were collected from the anesthetized animals after decapitation, frozen on dry ice and stored at -80C.
2) In the previous report immunohistochemical staining was used to detect the graft and to evaluate the levels of some of the pain- related markers. The distribution of graft is currently analyzed. Focus of this period was on biochemical evaluation of pain related biomarkers. We have evaluated the level of IL-1β, TNFα and IL-10 in the homogenates of the lumbar spinal cord from CCI and SCI animals from each treatment group.
Spinal cord frozen samples were homogenized in RIPA buffer (Santa Cruz) and the protein level was measured by BCA method (ThermoFisher). For ELISA detection of cytokines, kits for IL-1β, TNFα and IL-10 were used following the manufacturer protocol. Briefly, samples diluted to the same concentration of proteins were loaded onto 96 well plate in triplicates, together with standards, incubated with the blocking serum, followed by primary and secondary antibodies. Plates were read by a microplate reader (Molecular Devices) and results were analyzed by SotfMax Pro.

Our results show that the level of proinflammatory cytokines IL-1β and TNFα were reduced in both CCI and SCI animals treated with NPC or rNPC compared to control groups ($p<0.05$). Differences between NPC and rNPC groups were detected for IL-1β levels in CCI animals. Anti-inflammatory IL-10 showed similar levels between groups (Fig. 6)

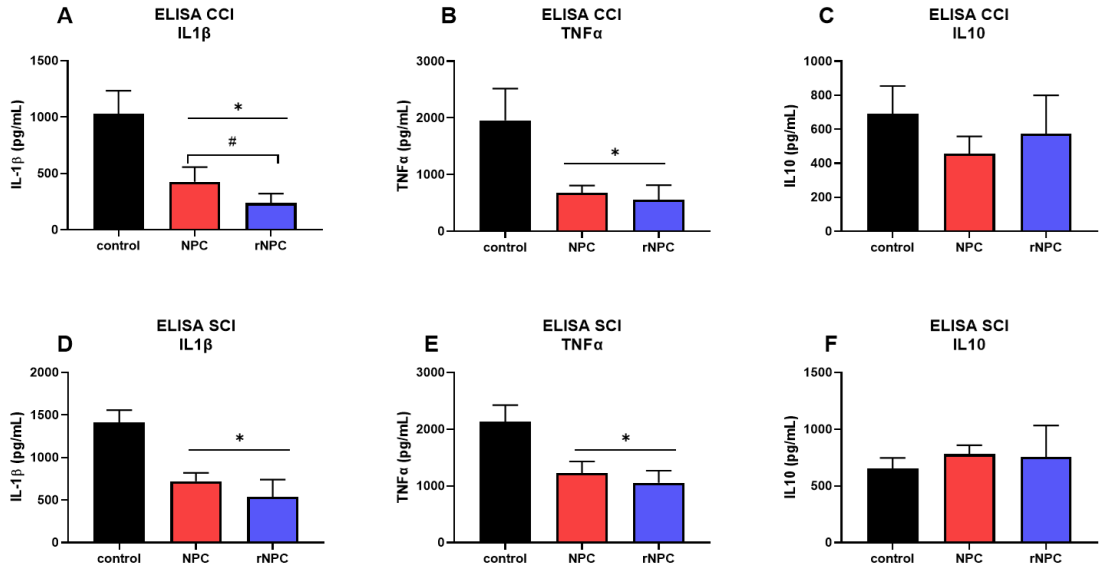

Fig. 6: ELISA quantification of A, D) IL-1β, B,E) TNFα and C, F) IL-10 in lumbar spinal cord homogenates in CCI and SCI animals with different treatment. *p<0.05 vs control, #p<0.05 between grafted groups.

To examine a relationship more closely between the level of pain-related cytokines and the behavior, we have run a correlation analysis test (GraphPad Prism) using data from ELISAs in combination with pain scores for each animal involved in the ELISA evaluation.
In CCI animals (males), significant correlations between behavior and the cytokines was detected for heat hypersensitivity and the level of IL-1β and TNFα. There was a trend for a positive correlation for tactile and cold hypersensitivity tests, but the values did not reach statistical significance (Fig. 7).
Much stronger relationships were observed in SCI animals (males), where correlation was detected for IL-1β and TNFα with the three behavioral tests. The level of IL-10 was comparable between groups with no significant correlation (Fig. 8)

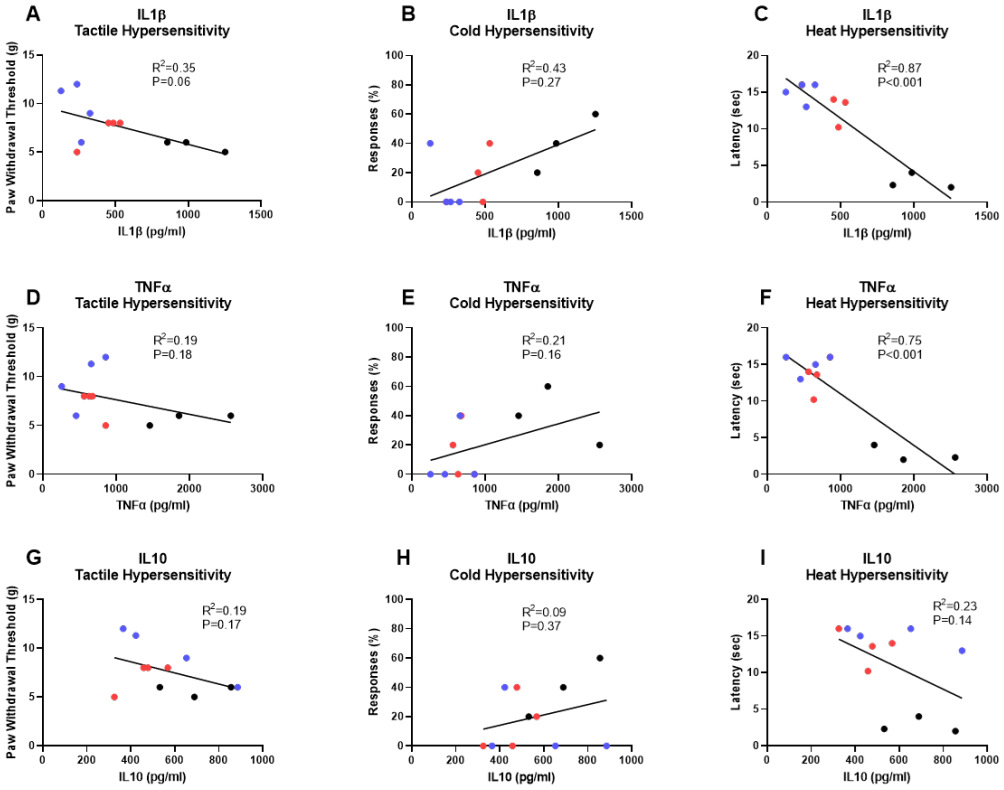

Fig. 7: Correlation analysis of the level of cytokines A-C) IL-1β, D-F) TNFα, G-I) IL-10 and behavior scores for tactile, cold and heat hypersensitivity in CCI animals treated with saline (black dots), NPC (red dots) or rNPC (blue dots). P values are indicated.

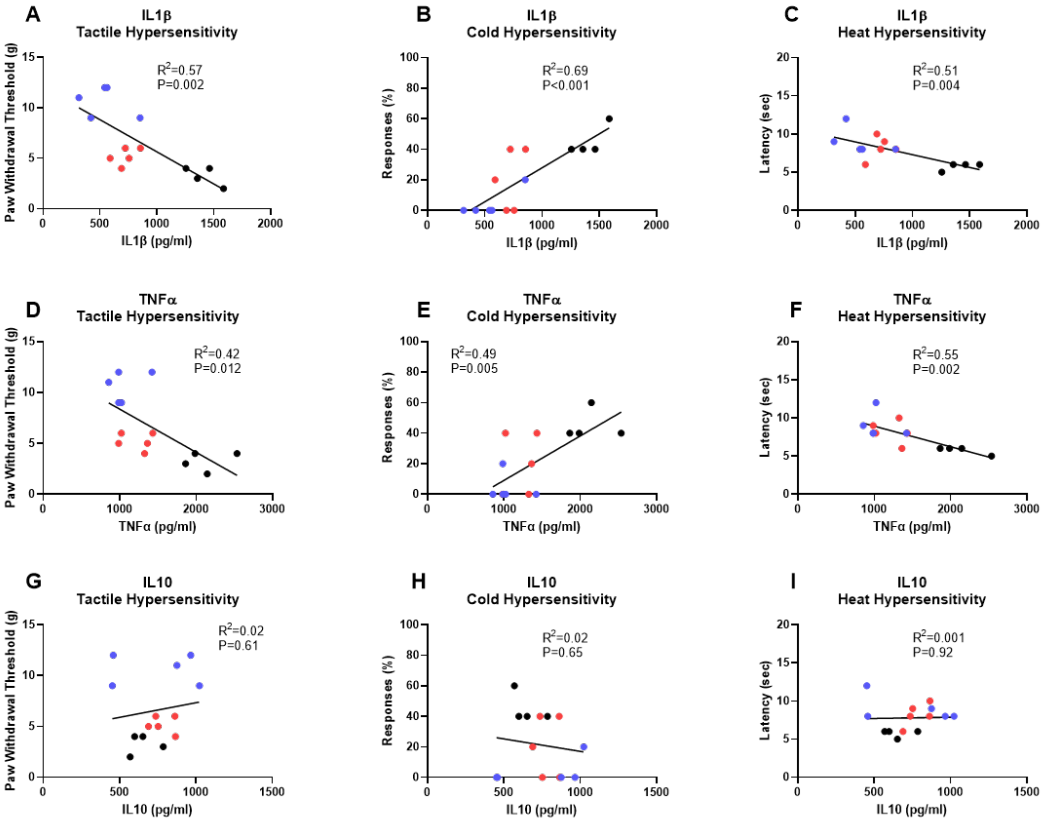

Fig. 8: Correlation analysis of the level of cytokines A-C) IL-1β, D-F) TNFα and behavior scores for tactile, cold and heat hypersensitivity in SCI animals treated with saline (black dots), NPC (red dots) or rNPC (blue dots). P values are indicated.

4. IMPACT

What was the impact on the development of the principal discipline of the project?
Nothing to report

What was the impact on other disciplines?
Nothing to report

What was the impact on the technology transfer?
Nothing to report

5. CHANGES/PROBLEMS

Changes in the approach and reason for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
General delays in experiments due to Covid-19 restriction in our facilities and in the material availability has been partially resolved and allowed us to continue in the experiments as planned using the non-cost extension period. Minor issues, such as limited availability of specific services to run RNA detection of MVIIA in samples as planned were overcome by using other methods to detect MVIIA in the recombinant cells and in vivo, both immunohistochemical and pharmacological.

Changes that had significant impact on expenditures
Nothing to report

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
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use biohazard and/or select agents
Nothing to report

6. PRODUCTS
Publications, conference papers and presentations
Due to Covid-19, several planned conferences have been canceled. Preliminary data were presented at Society for Neuroscience meeting (Marin et al, 2021) and are prepared for International Association for the Study of Pain conference (Jergova et al., 2021). Draft of the manuscript is in preparation as well.

Websites or other Internet sites
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name: Stanislava Jergova, PhD
Project Role: Principal Investigator
Researcher Identifier:
Nearest person month worked: 7
Contribution to Project: Dr. Jergova participated in the project design, training of personnel, recombinant cells engineering, surgeries and behavioral evaluations, tissue analysis, data management, preparation of reports, presentations and manuscript.
Funding Support : N/A

Name: Jacqueline Sagen,PhD
Project Role: Collaborator
Researcher Identifier:
Nearest person month worked: 1
Contribution to Project: Dr. Sagen managed animal protocols and supervised preparation of reports, presentations, and manuscript draft.
Funding Support : N/A

Name: Melissa Hernandez, MS
Project Role: Research Associate I
Researcher Identifier:
Nearest person month worked: 4

Contribution to Project: Ms. Hernandez provided surgeries and behavioral evaluation, participated on data evaluation and preparation of presentations and manuscript draft.
Funding Support : N/A

Name: Anjalika Eeswara, MS
Project Role: Research Associate I
Researcher Identifier:
Nearest person month worked: 4
Contribution to Project: Ms. Eeswara provided pre- and post-surgical treatments, surgeries and behavioral evaluations, tissue processing and managed data input.
Funding Support : N/A

Name: Barbara Marin
Project Role: Research Associate I
Researcher Identifier:
Nearest person month worked: 2
Contribution to Project: Ms. Marin assisted with pre- and post-surgical care of animals, behavioral evaluations, and tissue processing, data management and preparation of presentations and manuscript draft.
Funding Support : N/A

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS
Quad chart is attached

9. APPENDICES
Abstract SFN and Abstract IASP