AWARD NUMBER: W81XWH-19-1-0042

TITLE: Recombinant GABAergic Cells as a Therapy for Chronic Neuropathic Pain

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REPORT DATE: February 2022

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
February 2022	Final	15Apr2019-14Oct2021

4. TITLE AND SUBTITLE

Recombinant GABAergic Cells as a Therapy for Chronic Neuropathic Pain

5a. CONTRACT NUMBER
W81XWH-19-1-0042

5b. GRANT NUMBER
PR 182408

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER
W81XWH-19-1-0042

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

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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 project, 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: 100%

What was accomplished under these goals?

Major Task 1: IACUC and ACURO approvals

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) 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 $1x10^6$cells/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 $5x10^5$/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; VGAT, 1:200, Abcam; βTubulin 1:1000, Sigma; Doublecortin, 1:1000, Sigma; GFAP, 1:1000, Abcam; 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).

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.

Transduction efficiency was estimated at 83.6% of β tubulin+ cells (Fig. 1). No MVIIA signal was detected in non- β tubulin$_+$ cells. In general, no significant morphological changes were detected between recombinant and non-recombinant cells, as shown on Fig.1. Detail of GABA$_+$ recombinant cells are shown in Fig. 2. Cells from both groups shows GABAergic phenotype and form neurospheres.

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. 3).

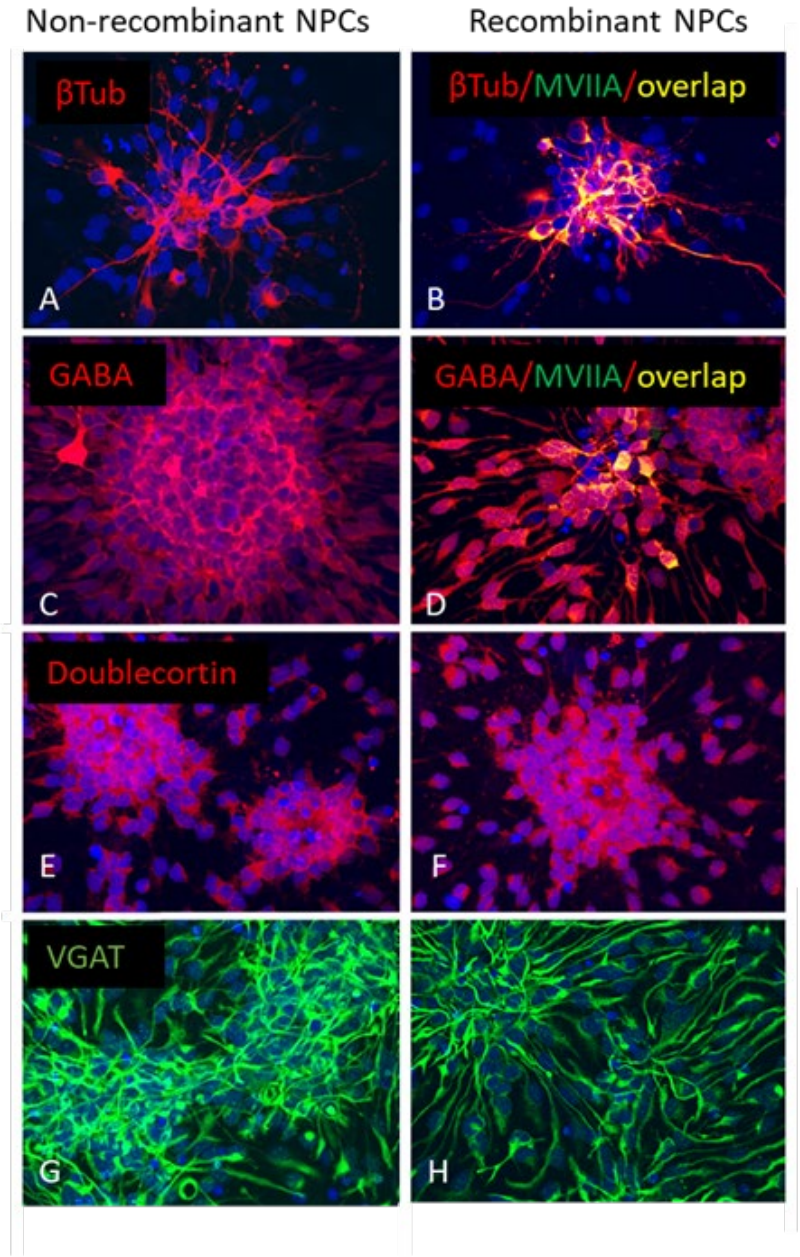
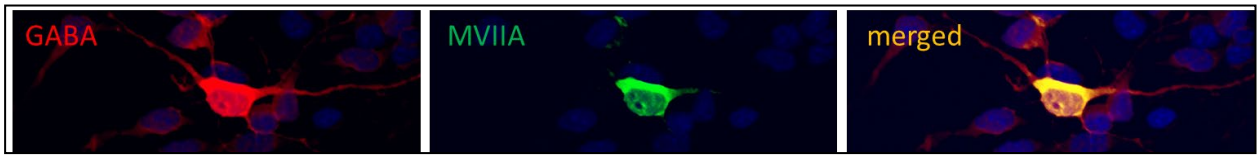


Fig. 1: Immunocytochemical detection of proneuronal markers and recombinant peptide MVIIA in E14.5 neuronal progenitor cells (NPCs).
A) B tubulin indicates proneuronal phenotype of cells.
B) Overlap between red βtubulin and green MVIIA creates yellow color in co-expressing cells.
C-D) GABAergic phenotype was confirmed in both non-recombinant and recombinant cultures.
E-F) Doublecortin is another proneuronal marker. Single staining was performed due to cross-reaction with MVIIA antibody. Morphology of the cells was not affected by the transfection.
G-H) VGAT is an early marker of GABAergic phenotype. Single staining due to cross-reaction. Similar cells morphology between cultures.

Fig. 2: Details of GABAergic cell expressing MVIIA. Characteristic morphology with long processes or more pronounced axon with dendrites indicates the overall "healthy" conditions of the recombinant cells.



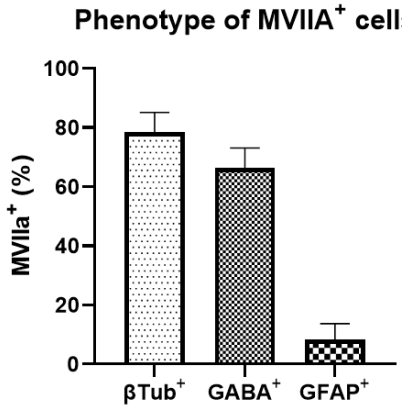
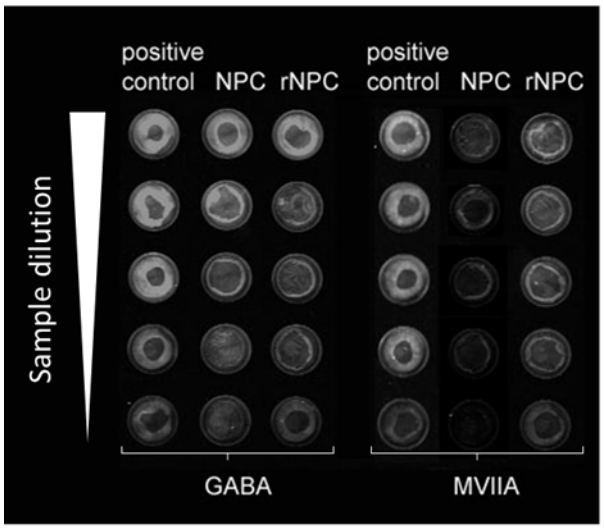
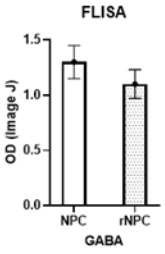


Fig. 3: Phenotype of MVIIA+ cells

4) To detect recombinant MVIIA peptide, FLISA analysis and immunoblotting were used. For FLISA, culture supernatants or peptides were coated onto wells in a 96-well format, incubated with anti-MVIIA and anti-GABA antibodies according to manufacturer's protocol. Wells were probed with anti-mouse and anti-rabbit antibody conjugated to IRDye 700 and 800CW and read using Odyssey Infrared Imaging System. For immunoblotting, samples were loaded on gradient gel, run at 100V for 1.5h, and transferred on PVDF membrane (Immobilon PSQ; Millipore). Blots were incubated in MVIIA, mRFP and β-actin primary antibodies (rabbit anti-mRFP 1:1000 Chemicon; mouse anti-beta-actin 1:1000 Abcam; anti-MVIIA monoclonal antibody 1:1000, 21st Century Biochemicals), followed by horseradish peroxidase (HRP) conjugated secondary antibodies (1:10000 goat anti-rabbit HRP; Santa Cruz; 1:5000 goat anti-mouse IgG-HRP) and detected by chemiluminiscence (Perkin-Elmer) with BioRad FluorS scanner equipped with Quantity One software. For western blot, samples were loaded onto agarose gel, run at 100V for 2 hours and transferred to membrane. Membrane was incubated in the primary MVIIA and GAPDH.

Results of FLISA analysis shows the presence of GABA in the non-recombinant and recombinant cells as expected and the presence of MVIIA peptide in the supernatant of recombinant cells (Fig. 4). Immunoblot analysis was under the detection range of the scanner, the size of the MVIIA peptide was also an issue with the immunoblot detection, FLISA detection proved to be sufficient.





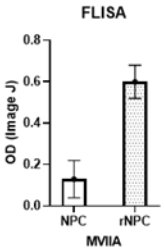


Fig. 4: FLISA analysis of cell culture supernatant for the presence of GABA and MVIIA. GABA was detected in both non-recombinant and recombinant cells cultures, MVIIA signal was significantly different from the background in recombinant cells supernatant samples.

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) Ms. Hernandez has previous experiences with the model, a brief training was introduced to assure she can perform all surgical and behavioral procedures according to the approved protocols. To induce CCI, adult male Sprague-Dawley rats weighing 200-300 g were used. Animals are anesthetized with 2-3% isoflurane in O_2, and the common sciatic nerve exposed on one side at the mid-thigh level using aseptic surgical techniques. Four 4-0 chromic gut ligatures spaced about 1 mm apart are loosely tied around the sciatic nerve proximal to the trifurcation. Following surgery, the musculature is sutured in layers, and the skin closed with wound clips.

2) 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 1 week post CCI to target early stages of chronic pain development in this particular model. Cells were injected in the ipsilateral 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 $x10^4$ cells) was stereotactically injected on the injury side. 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 the surgery.

3) Behavioral tests:
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.

CCI induced development of hypersensitivity to mechanical and thermal stimulation within the first week post injury. Alleviation was observed in animals with grafts. In the CCI model there is a spontaneous recovery observed after 2 months post injury and animals might show less sensitivity. Cells were grafted at 1 week post CCI when the hypersensitivity was already developed.

Fig. 5A: Saline injected animas (controls) developed signs of tactile hypersensitivity that persisted throughout the experiment. Animals with non-recombinant graft showed mild attenuation of hypersensitivity, with significant difference from the control group starting at 3 weeks post grafting (week 4) till the week 9 ($p<0.05$). Animals with recombinant cells showed better outcomes, when the effect was significantly different from the non-recombinant graft at weeks 7-12 ($p<0.05$). The withdrawal threshold of animal in this group reached almost the pre-injury levels at the end the experiment.

Fig. 5B: Saline injected animals developed cold hypersensitivity with a peak between weeks 4-7 post injury. Non-recombinant grafts prevented development of severe hypersensitivity, with significantly lower response rate compared to control animals starting at 3 week post grafting and lasting till the end of experiment ($p<0.05$). Recombinant grafts further attenuated ongoing hypersensitivity, reaching almost the pre-injury level at 8 weeks post grafting. The overall cold responses were lower than in control group starting at 2 weeks post grafting and were lower compared to the non-recombinant group between weeks 5-7 ($p<0.05$).

Fig. 5C: Saline injected animal progressively developed hypersensitivity to noxious heat stimulation that remained stable thought the experiment. Non-recombinant grafts attenuated heat hypersensitivity starting at 2 weeks post grafting with statistically higher latency compared to the control animals throughout the experiment ($p<0.05$). Recombinant grafts attenuated heat hypersensitivity starting at 2 weeks post grafting with significantly better outcome compared to both control ($p<0.01$) and non-recombinant ($p<0.05$) grafted groups starting at week 7 and lasting through the remainder of the study.

In general, recombinant graft showed stronger and longer lasting pain attenuation effect compared to non-recombinant graft.

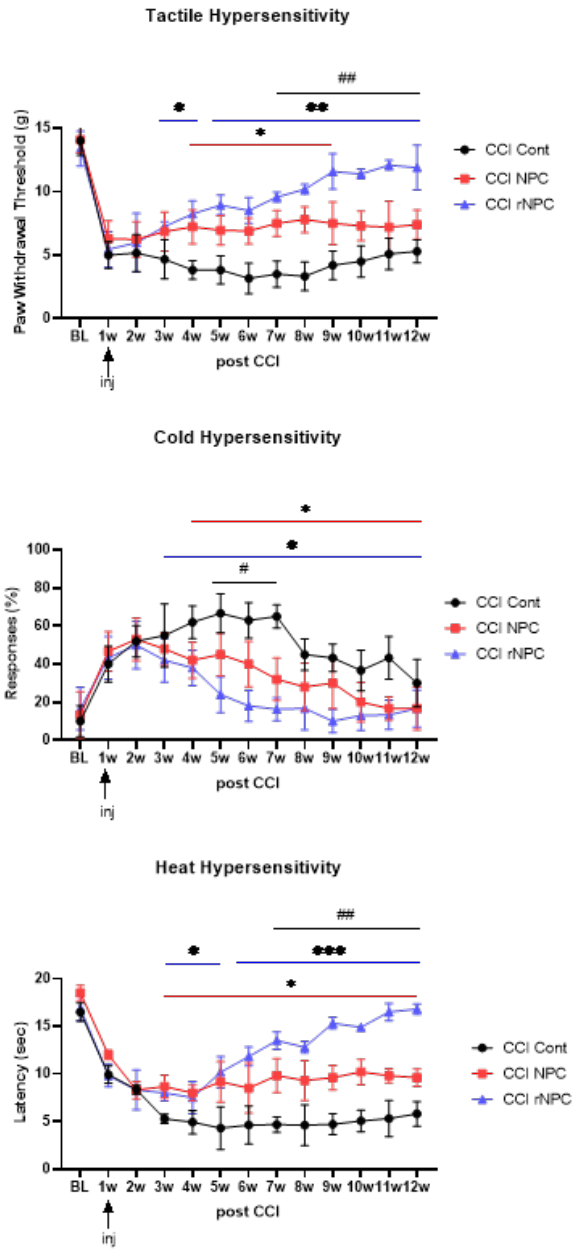


Fig. 5: Behavioral evaluation of the cell therapy in CCI model. *p<0.05, **p<0.01, ***p<0.001 vs control (color coded); #p<0.05, ##p<0.01 between groups.

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 expose 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.

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 x10^4 cells) was stereotactically injected on each side. Following transplantation, the area was covered with elastic sheathing, the overlying musculature sutured, and the skin closed with wound clips. Control animals received equal volume of saline injection. Animals with grafted cells received daily cyclosporine injections starting 2 days prior to the surgery.

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

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 was 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.

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 in the dark side was considered as a positive outcome of the therapy.

Males (Fig. 6): 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 for both NPC groups starting 2 weeks post injection (7w post injury) and continued 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, $p<0.05$), 2-5 weeks post injection for cold (B, $p<0.05$) and 2-10 weeks post injection for heat (C, $p<0.05$) hypersensitivity. Locomotor scoring using BBB scale was not significantly different overall, but there were subtle changes between animals with rNPC and control group, statistically significant between 2-4 weeks post injection.

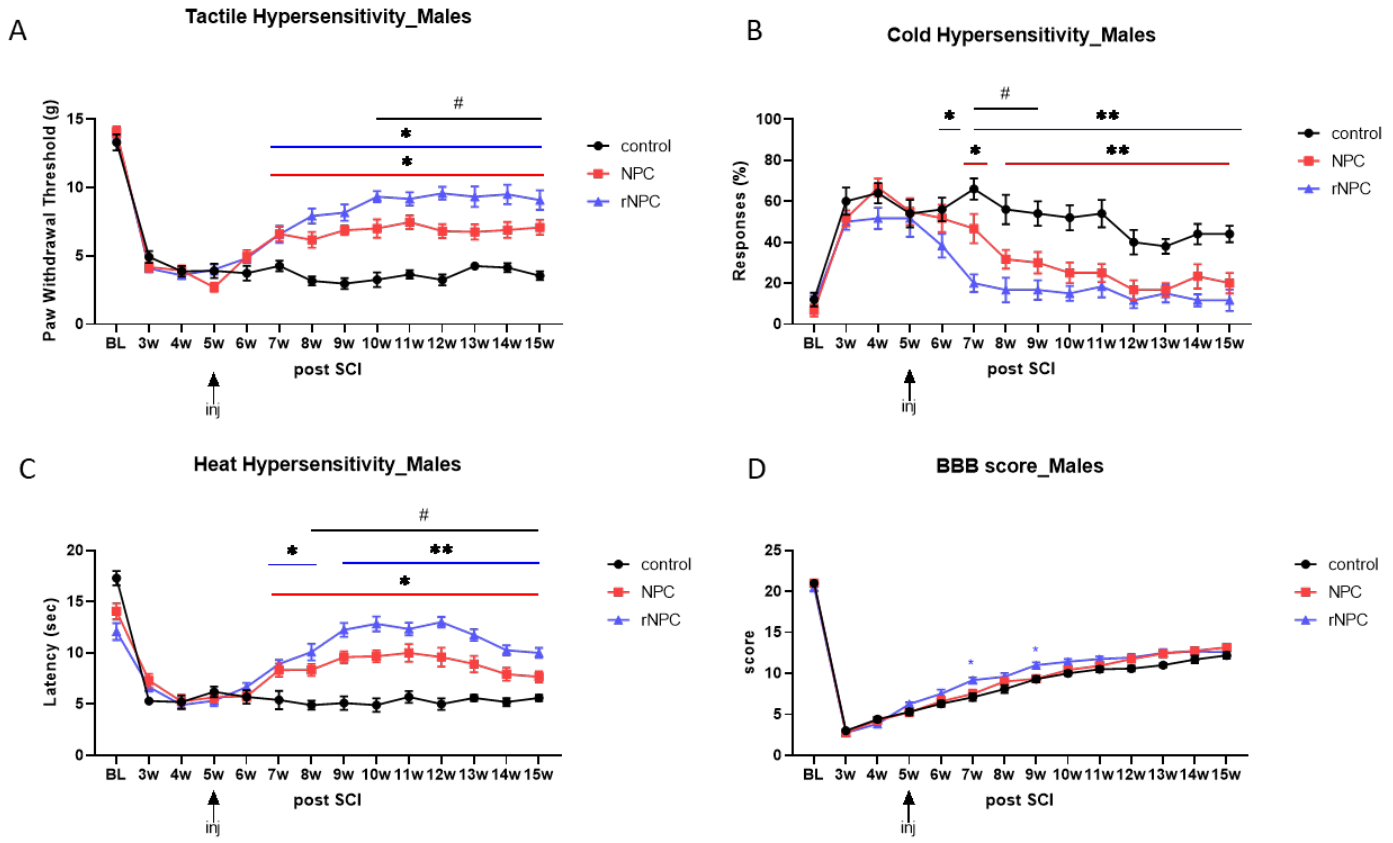


Fig. 6: Behavioral evaluation of A) tactile, B) cold , C) heat hypersensitivity and D) locomotor score in SCI male rats with different treatment. *p<0.05, **p<0.01 vs control (color coded), #p<0.05 between grafted animals.

Females (Fig. 7): Behavioral responses of female rats were 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 treated animals, although a partial drop in cold hypersensitivity was observed 15 weeks. NPC and rNPC reduced hypersensitivity compared to control animals starting at 2 weeks post injection. Significant 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 weeks 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 with faster regain of voiding (within 3-5 days post injury).

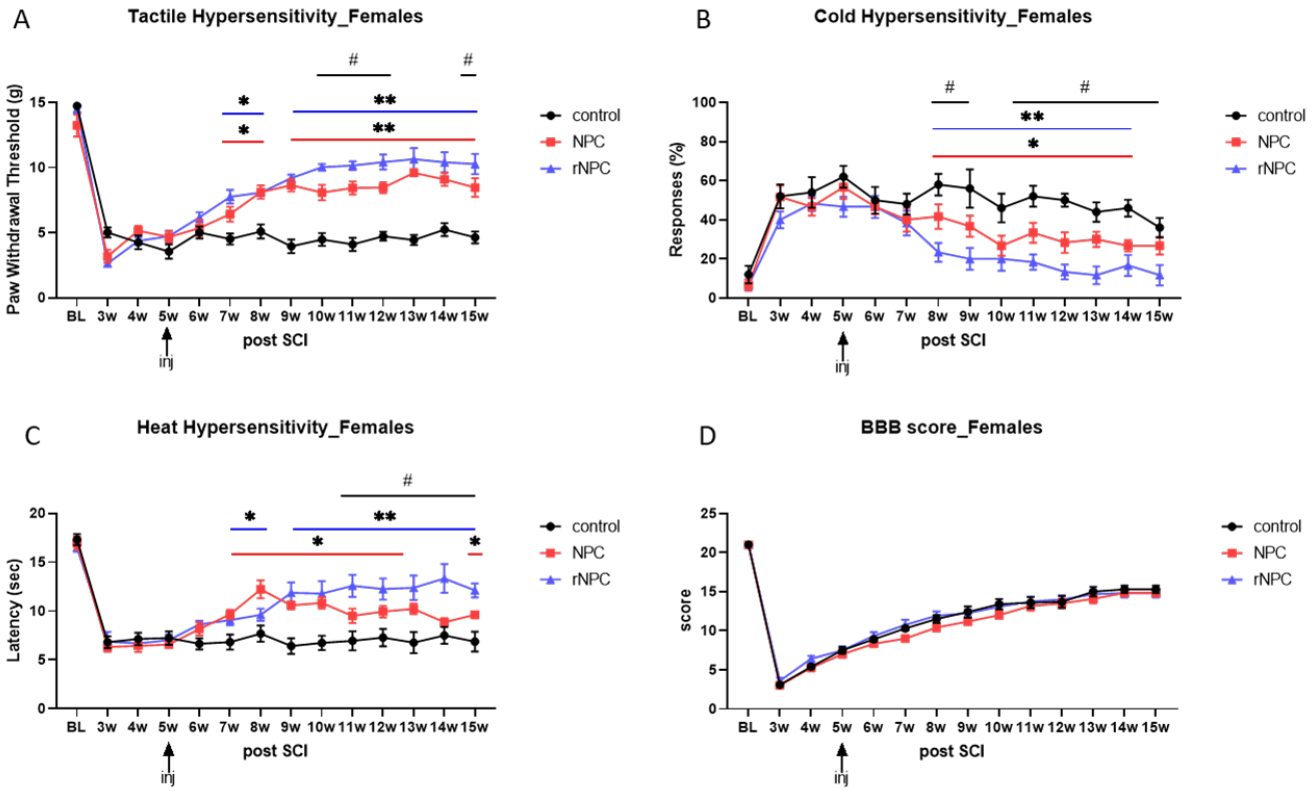


Fig. 7: Behavioral evaluation of A) tactile, B) cold , C) heat hypersensitivity and D) locomotor score in SCI female rats with different treatments. *p<0.05, **p<0.01 vs control (color coded), #p<0.05 between grafted animals.

To further evaluate the analgesic effects of the treatments, 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 done to pair with the preferred side of the cage. This avoidance suggests the presence of ongoing pain. NPC and rNPC treated animals did not show significant differences (avoidance) in the time spent in the preferred side, despite the presence of tactile stimuli (Fig. 8).

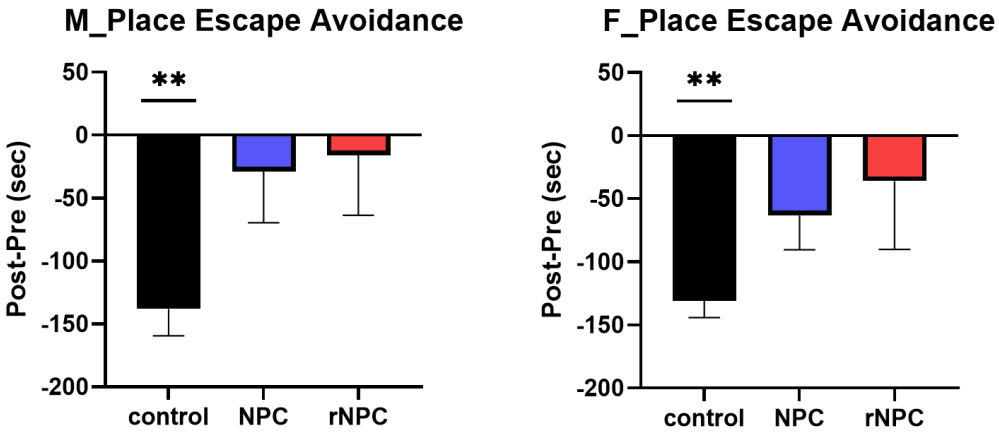


Fig. 8: Place escape avoidance behavior. Control, saline treated animals display avoidance behavior-less time spent on the preferred side of the cage in the presence of a tactile stimuli. **p<0.01 vs null hypothesis

The analgesic effect of MVIIA released from the recombinant cells was evaluated in the tactile and cold hypersensitivity tests in animals with recombinant graft using intrathecal injection of MVIIA antibody. To insert the catheters, 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 exposed, and a 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. 5µl of MVIIA antibody (2µg/kg, 21st Century Biochemicals) followed by 5µl 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 in hypersensitivity scores. Injection of MVIIA antibody led to reduction of analgesic effects of the recombinant graft treatment and increase of hypersensitivity in both tests and in both sexes (at least $p<0.0$ compared with pre-injection). Female animals responded even more robustly to MVIIA antibody injection in the cold hypersensitivity test with $p<0.001$ (Fig. 9).

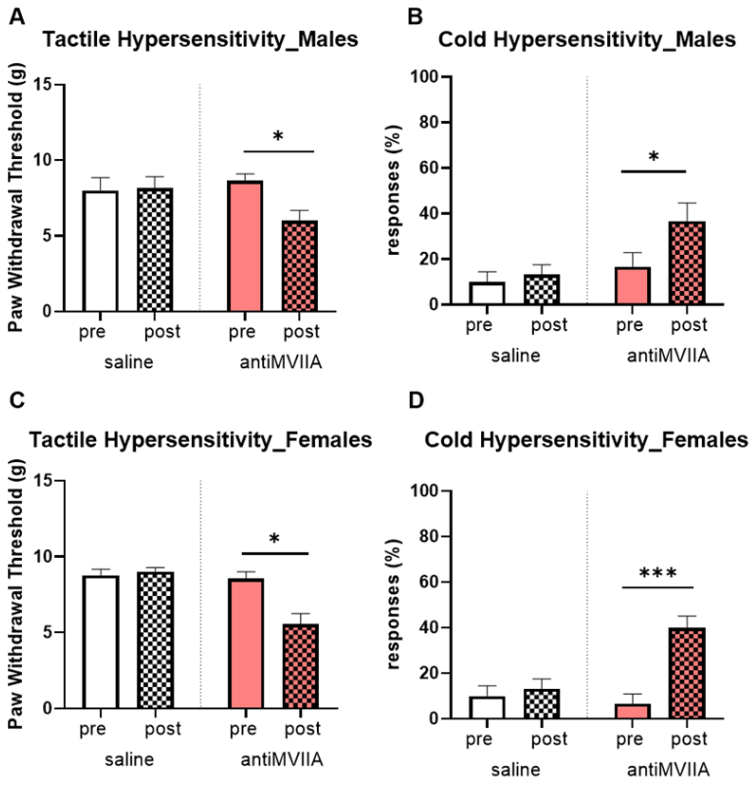


Fig. 9: Intrathecal injection of MVIIA antibody reduced analgesic effects of the recombinant graft evaluated for A,C) tactile and B,D) cold hypersensitivity in male and female animals. *p<0.05, ***p<0.001.

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 tests are finalized and to prepare the tissue for biochemical or histochemical analysis.
2) To evaluate the distribution, survival and the physiological effects 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) Immunohistochemical staining was used to detect the grafts and to evaluate levels of cellular phenotypes and some pain-related markers. Sections were incubated in 5% normal goat serum, followed by overnight incubation in primary antibodies in 4°C (recombinant conopeptide MVIIA 1:200 (21st century Biochemicals); gama-ammino-butyric acid (GABA) 1:200 (Sigma); Neuronal N (NeuN) 1:1000 (Genetex); glial fibrilary acidic protein (GFAP) 1:1000 (Millipore); Iba-1 1:1000 (Wako), Calcitonin gene related peptide (CGRP, 1:1000, Millipore)) and incubations in secondary antibodies (anti-rabbit, anti-mouse, anti-guinea pig, 1:200, Alexa Fluor) in 5% NGS. Sections are glass mounted, air dried and coverslipped by VectaShield (Vector Labs). Analyses is done with a Zeiss Axiovert 200M research microscope (Ludl Electronic Products), DVC cooled camera and multi-band fluorescent filters allowing for viewing single, double or triple fluorophores at the same time.

We have observed reduction in the amount of GABA+ cells in the spinal dorsal horn in the control CCI animals (Fig. 10A-D). Grafting of recombinant or non-recombinant cells partially restored the amount of GABA+ cells (white arrows in Fig. 10 ADC). We have analyzed possible changes in the level of CGRP, as changes in the expression of this pain-related neurotransmitter are documented in several studies. We observed a reduction in the immunostaining in the middle dorsal horn area in the control animals but not significantly different from the grafted groups (Fig.10 E-H). Similarly, activation of microglial cells detected by Iba-1 imunostaining is observed in CCI model early post injury. However, at 12 weeks post CCI in the current study, no significant differences were detected between the treatment groups (Fig.10 I-L).

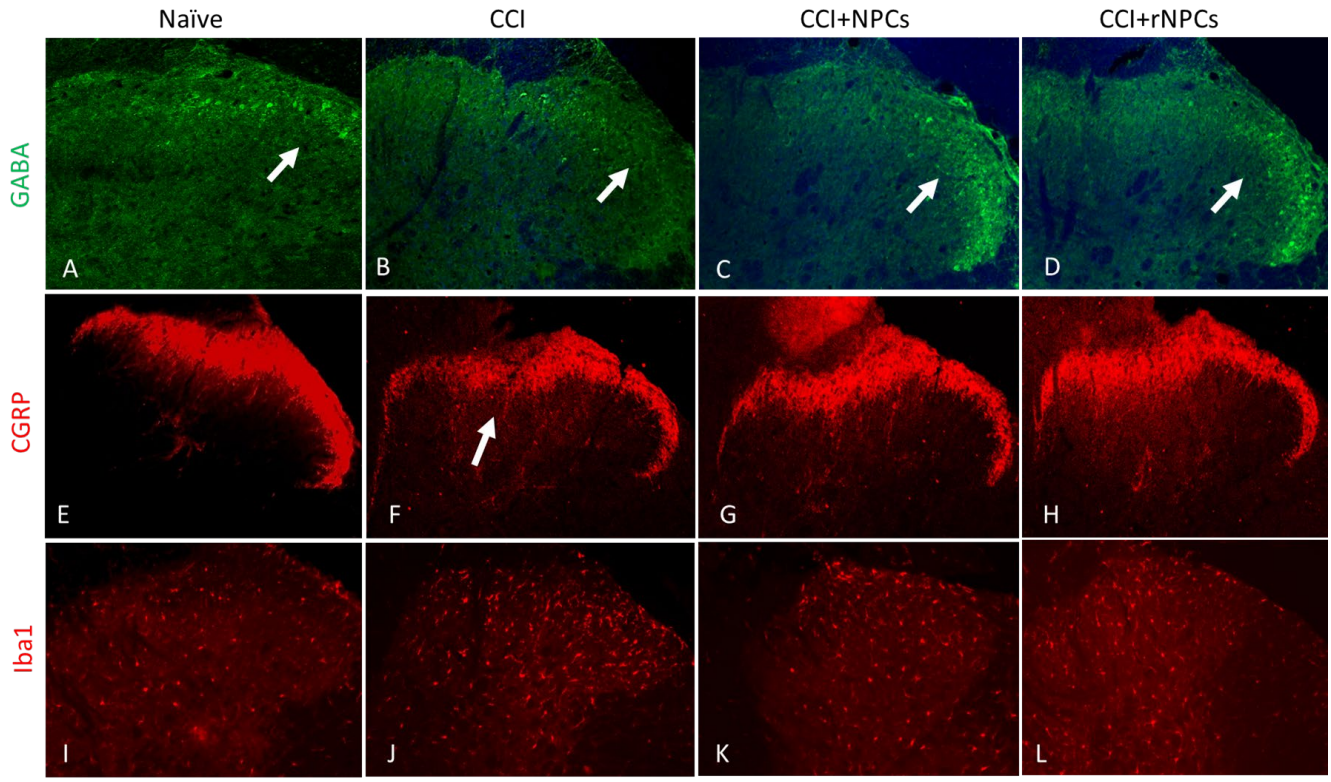


Fig. 10: Immunohistochemical detection of GABA (A-D), CGRP (E-H) and Iba1 (I-L) in the spinal dorsal horn of animals with intraspinal saline (control), non-recombinant and recombinant cell graft. Tissue was analyzed at the end of experimental period (12 weeks post CCI). Reduced amount of GABA+ cells observed in control CCI animals was partially restored after cell graft (A-D). Changes in the level of CGRP were only minimal. Microglial activation was similar between groups.

Recombinant grafted cells were detected in the lumbar spinal cord with minimal spreading from the injection site (about 0.8mm rostral and caudal, Fig. 11). Grafted cells show predominantly a neuronal phenotype (NeuN). In the analyzed tissue we have not found overlap of the MVIIA signal with the glial marker GFAP.

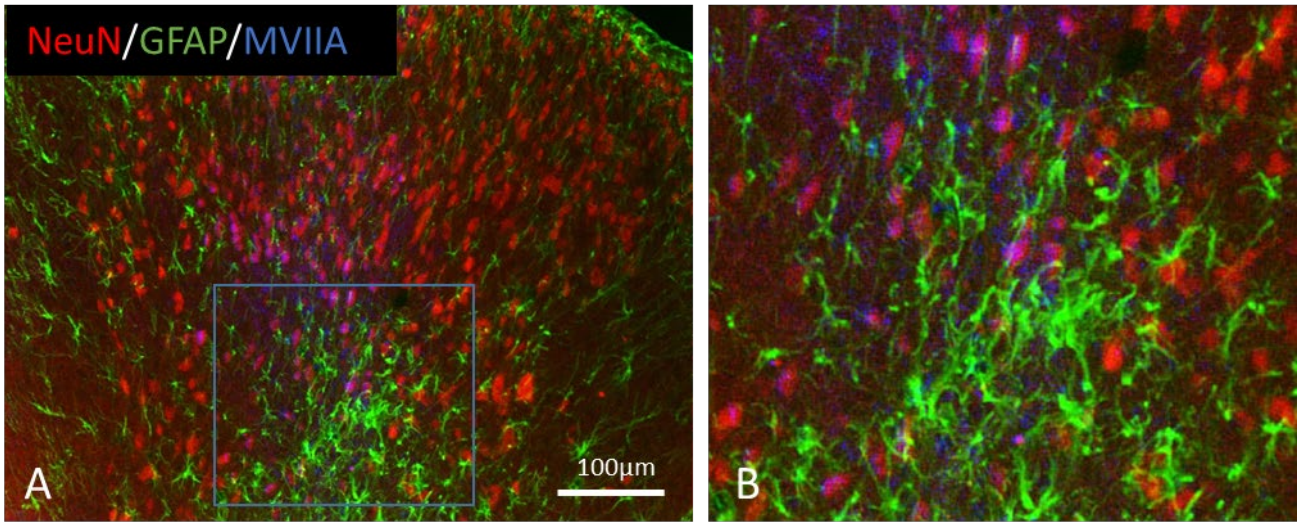


Fig. 11: A) Recombinant cells detected in the spinal dorsal horn. NeuN (red) labels neuronal cells, GFAP (green) astrocytes and MVIIA (blue) labels cells producing MVIIA protein. Overlap between NeuN and MVIIA appears as magenta. No overlap between GFAP and MVIIA was observed. B) Enlarged area with the graft.

Biochemical evaluation of the effect of grafted cells was conducted by ELISA analysis of pain related cytokines in the spinal cord homogenates. 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 the 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 SoftMax Pro.

CCI:

Our results show that the level of proinflammatory cytokines IL-1β and TNFα were reduced in CCI animals treated with NPC or rNPC compared to control CCI groups ($p<0.05$). More robust reduction of spinal IL-1β levels was observed in rNPC groups compared with non-recombinant NPCs. Anti-inflammatory IL-10 showed similar levels between groups (Fig. 12).

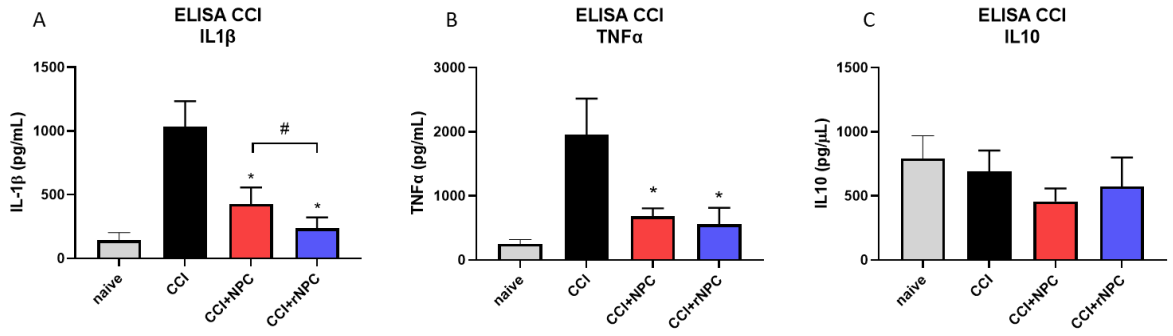


Fig. 12: ELISA evaluation of A) IL1β, B) TNFα and C) IL10 levels in the lumbar spinal homogenates in CCI animals. *$p<0.05$ vs CCI, #$p<0.05$ between indicated groups.

SCI:
In SCI animals, decreased levels of IL1b and TNFa were observed in NPC treated animals in both males and females ($p<0.05$), compared with saline treated SCI controls. Further reduction and significant decreased IL1b between NPC and rNPC treated animals was observed in females (#$p<0.05$). Also in this group, an upregulation of IL10 was observed in females treated with rNPC (#$p<<0.05$). This might account for sex-related differences in the pain processing, although the overall behavior was comparable between males and females (Fig. 12).

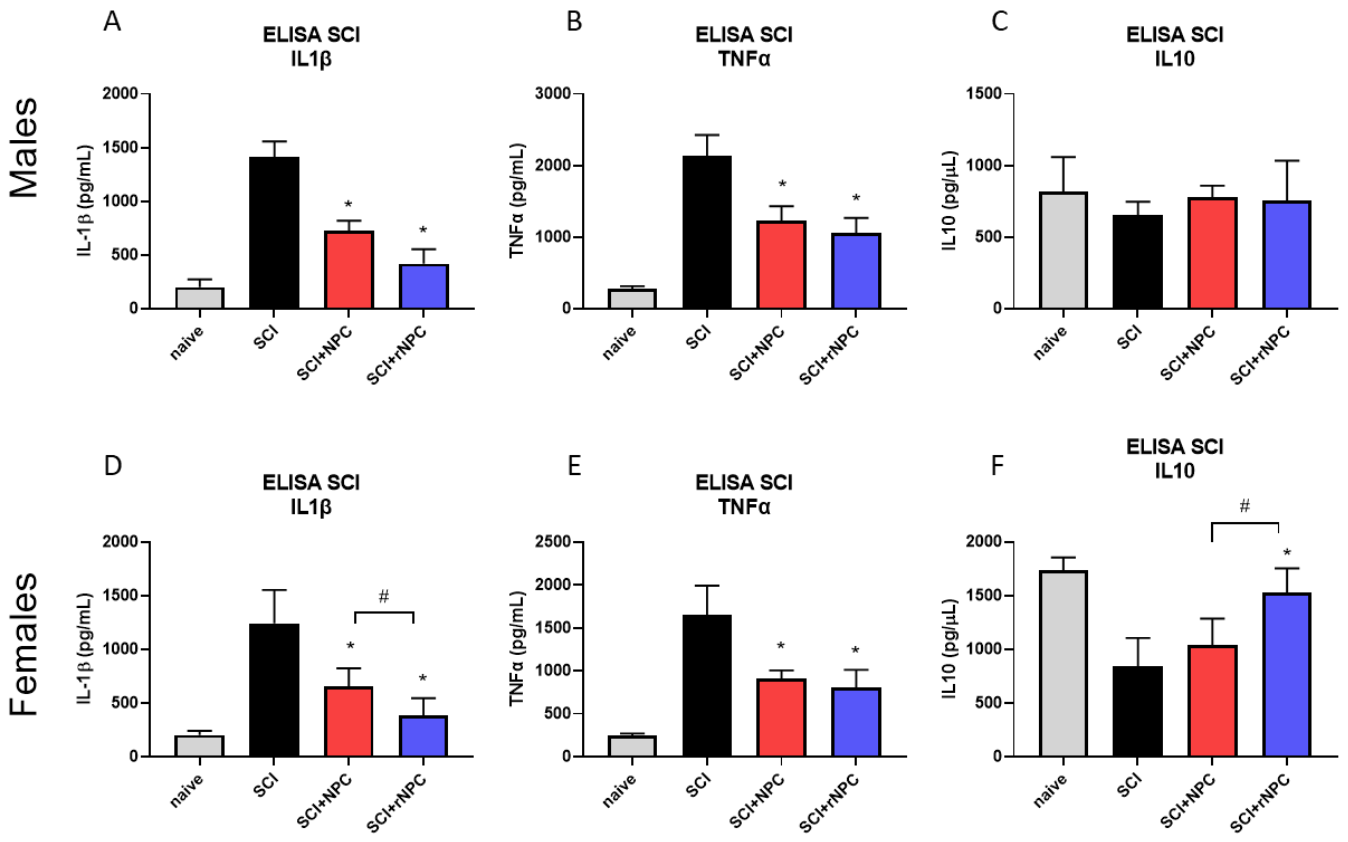


Fig. 13: ELISA evaluation of A, D) IL1β, B, E) TNFα and C, F) IL10 levels in the lumbar spinal homogenates in SCI males and females respectively. *$p<0.05$ vs SCI, #$p<0.05$ between indicated groups.

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 individual animals used in the ELISA evaluations.

In CCI animals (males), significant correlations between behavior and the cytokines was detected for heat hypersensitivity and the levels 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. 14).

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. 15)

Similar correlations were observed in the female groups, with significant values for all tested cytokines in each of the behavioral tests. (Fig.16).

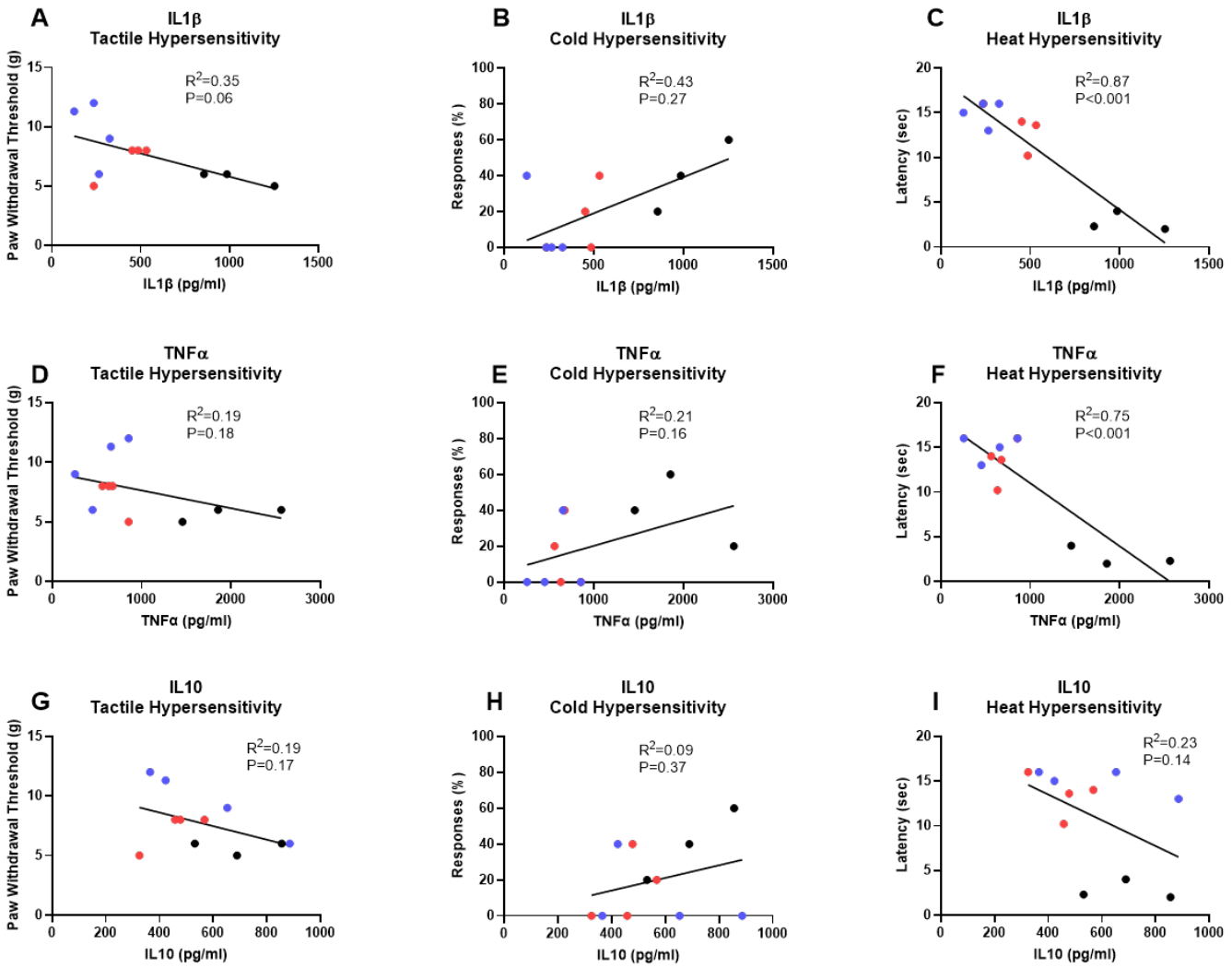


Fig. 14: Correlation analysis of the behavioral outcome and the level of cytokines in the spinal homogenates for IL1β (A-C), TNFα (D-F) and IL10 (G-I) and tactile (A,D,G), cold (B,E,H) and heat (C,F,I) hypersensitivity in CCI animals. P values are indicated.

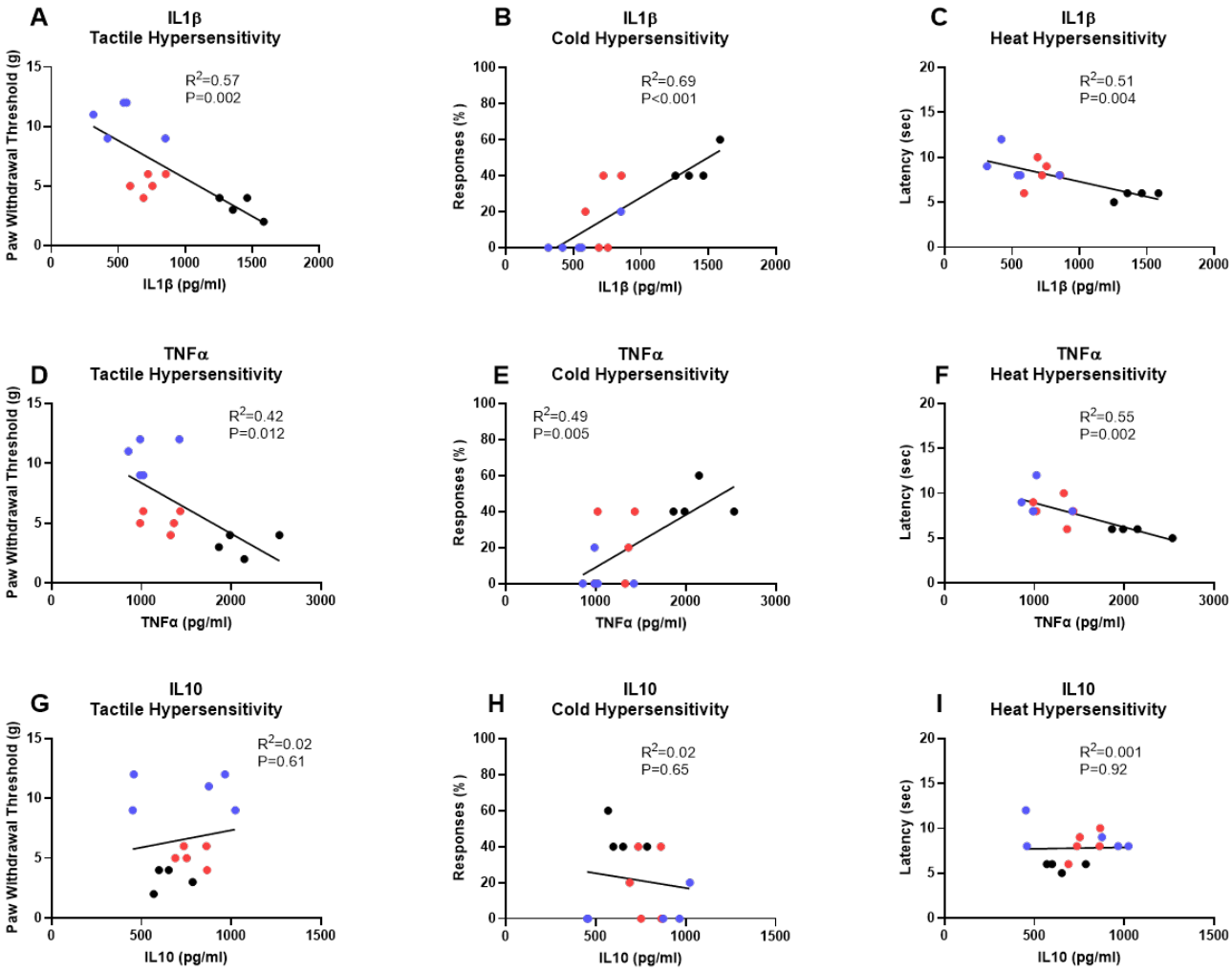


Fig. 15: Correlation analysis of the behavioral outcome and the level of cytokines in the spinal homogenates for IL1β (A-C), TNFα (D-F) and IL10 (G-I) and tactile (A,D,G), cold (B,E,H) and heat (C,F,I) hypersensitivity in SCI male animals. P values are indicated.

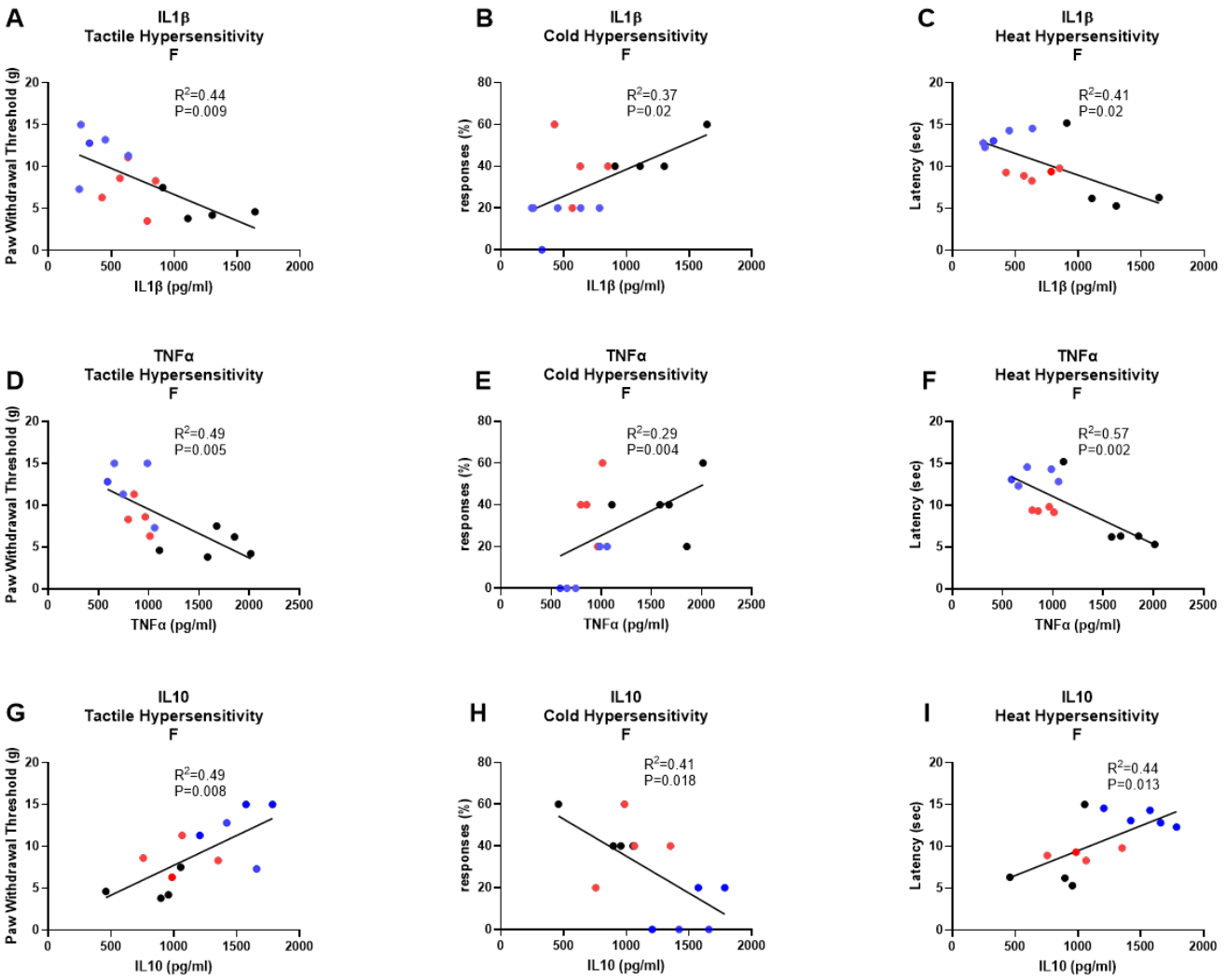


Fig. 16: Correlation analysis of the behavioral outcome and the level of cytokines in the spinal homogenates for IL1β (A-C), TNFα (D-F) and IL10 (G-I) and tactile (A,D,G), cold (B,E,H) and heat (C,F,I) hypersensitivity in SCI female animals. P values are indicated.

Histochemical analysis of the spinal tissue from SCI animals showed reduced level of immunostaining for the enzyme GAD65/67 that is involved in the production of GABA in the animals with SCI and a partial recovery of the signal in animals with grafted cells in both males and females (Fig. 17).

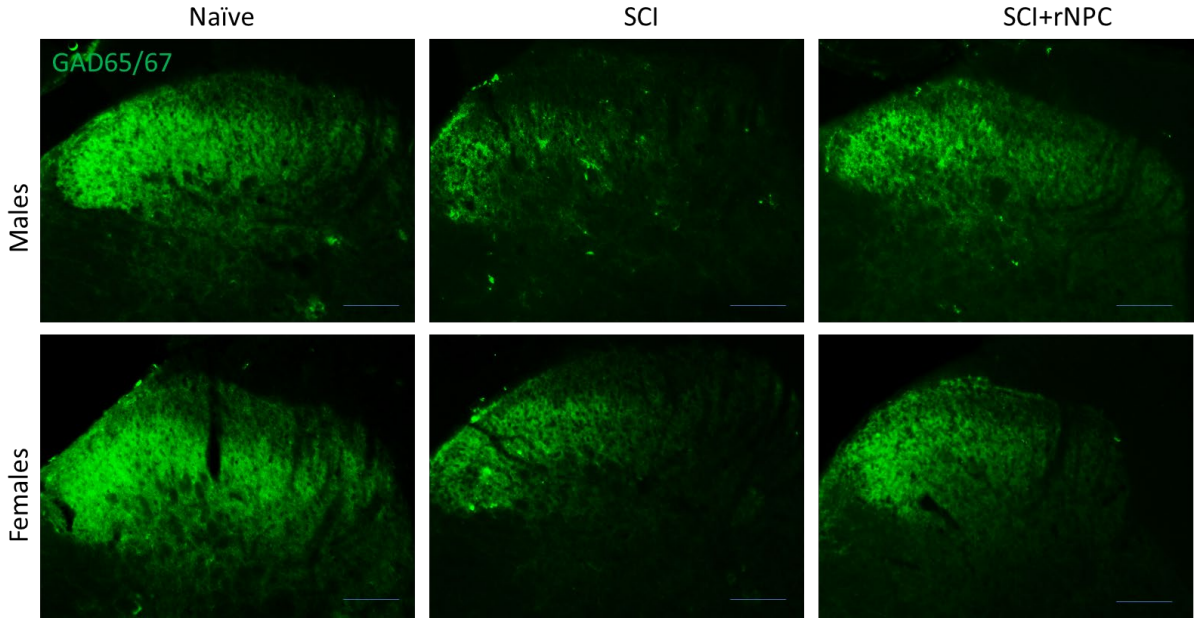


Fig. 17: Immunohistochemical visualization of GAD65/67 in the spinal dorsal horn in male and female animals. The level of GAD65/67 in SCI animals (middle panel) is significantly reduced compared to naïve (left panel). Partial restoration is observed in grafted animals (right panel).

Further biochemical analysis using the FLISA method showed decrease in GABA levels in the spinal homogenates of animals with SCI and its upregulation in grafted animals, confirming our findings observed with GAD65/67 immunostaining. (Fig. 18). The presence of MVIIA has also been confirmed in the tissue of both males and females grafted with the recombinant cells. (Fig. 19).

Histological evaluation of lumbar spinal cord slides in animal grafted with recombinant cells confirmed the presence of recombinant GABA/MVIIA cells in the spinal dorsal horn of both male and female rats at 5 weeks post grafting when the effect of grafted cells was fully established, and also at the end of the experimental period (Fig. 20). Cells were detected within 1mm caudal and rostral from the injection point.

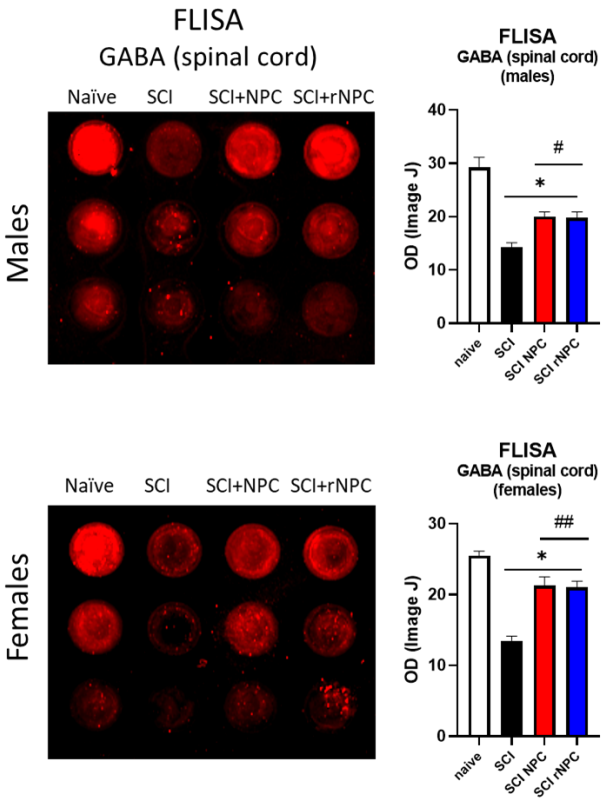


Fig. 18: FLISA analysis of GABA in naïve, SCI, SCI+NPC and SCI+rNPC males and females spinal homogenates. *p<0.05 vs naive, #p<0.05 vs SCI.

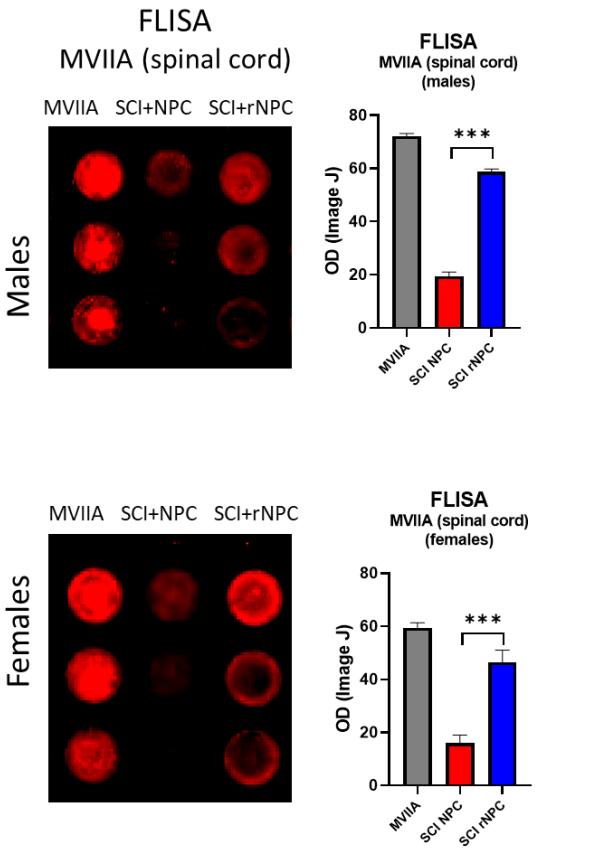


Fig. 19: FLISA analysis of MVIIA in spinal homogenates of SCI animals grafted with nonrecombinant and recombinant NPC. MVIIA peptide was loaded as a positive control. ***p<0.001 vs NPC.

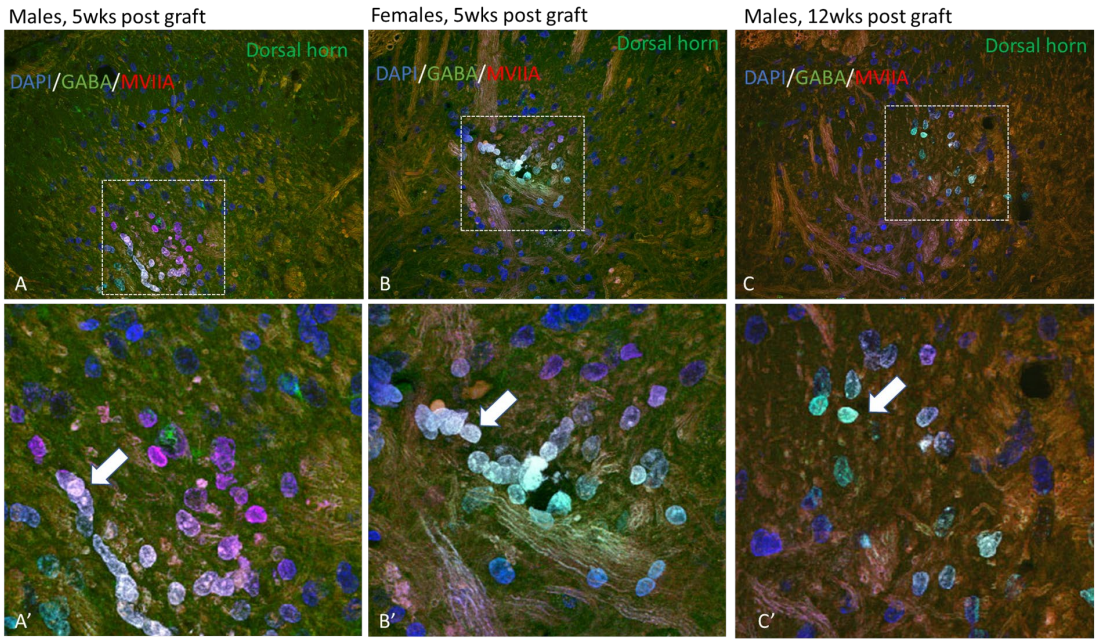


Fig. 20: Immunocytochemical staining of lumbar spinal slides for detection of GABA (green) and MVIIA (red) NPCs in males at 5 weeks post grafting (A), females at 5 weeks post grafting (B), and males at 12 weeks post grafting (C). Colocalization of GABA and MVIIA markers with DAPI nuclear staining (blue) yields white cells (e.g. arrows in A', B', C').

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 restrictions in our facilities and in the material availability occurred, but was primarily 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), at International Association for the Study of Pain conference (Jergova et al., 2021) and are prepared for presentation for the upcoming virtual Society for Neuroscience meeting (Liebmann, 2021). A decision was made to split the results into two manuscripts for CCI and SCI models, drafts are updated.

Websites or other Internet sites

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

University of Miami Technology Transfer Office will evaluate a potential need for disclosures

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: 13
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: 2
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: 11
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: 9
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: 3
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

Name: Kevin Liebmann
Project Role: Research Associate I
Researcher Identifier:
Nearest person month worked: 2
Contribution to Project: Mrs. Liebmann assisted with data analysis, image evaluation, data management and preparation of presentations.
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
Abstracts SFN and Abstract IASP