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TITLE: Enhancing Recovery of SCI-Induced Bladder Dysfunction Using Small Molecules

PRINCIPAL INVESTIGATOR: Anthony Kanai, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh

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

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Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The p75 neurotrophin receptor has been shown to be involved in various neurodegenerative conditions and our preliminary data indicate that it is also involved in bladder complications resulting from spinal cord injury (SCI). LM11A-31 prevents activation of degenerative signaling while promoting nerve regeneration pathways. Preliminary findings demonstrate that daily oral administration of LM11A-31 is the first agent that treats both neurogenic detrusor overactivity (NDO) and detrusor sphincter dyssynergia (DSD) in mouse models. These effects could be translated in SCI patients to decrease NDO, incontinence, catheterization, urinary tract infections, DSD and kidney damage to improve the health and quality of life of SCI patients. Accordingly, we propose to study the therapeutic benefit of LM11A-31 in male and female complete and partial SCI mice assessed using a range of physiological and molecular techniques. Both complete transection and contusion injuries will be evaluated as the pathological outcomes can vary significantly depending upon the extent of injury. Further, the neural regenerative properties of the tropomyosin related kinase receptor agonist, LM22B-10, will be evaluated in combination with the protective effect of LM11A-21. Our preliminary data demonstrate the efficacy of LM11A-31 treatment in complete SCI and we hypothesize that there will be even more beneficial effects in spinal contusion-induced bladder dysfunction.

15. SUBJECT TERMS: Spinal cord injury, proneurotrophins, p75 neurotrophin receptors, neurogenic bladder overactivity, detrusor-sphincter dyssynergia.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	30	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** ***Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.***

The project investigated the therapeutic potential of the p75 neurotrophin receptor modulator, LM11A-31 and the tropomyosin-related kinase (Trk)B/C receptor agonist LM22B-10 for amelioration of secondary damage following spinal cord injury (SCI). The study utilized severe thoracic level spinal cord contusion injury in a mouse model to determine if treatment with either drug or their combination would improve recovery of lower urinary tract function.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Spinal cord injury, proneurotrophins, p75 neurotrophin receptors, neurogenic bladder overactivity, detrusor-sphincter dyssynergia.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1: Determine the time window after SCI during which LM11A-31 can effectively treat DSD.

- Subtask 1: Obtain local IACUC approval and ACURO for animal studies. **Completed 07/23/18 and 10/29/18, respectively.**
- Subtask 2: Examine SCI-induced changes in voiding patterns by spot tests, metabolic cage assessments and CMG-EMG recordings. **Completed.**
- Subtask 3: Examine changes in urothelial transepithelial resistance (TER) following SCI. **Initiated, ~30% completed.**
- Subtask 4: Histological assessing of urothelial morphology and apoptosis following SCI. **Completed.**

Major Task 2: Compare the therapeutic benefits of LM11A-31 in treating bladder dysfunction in SCC and SCT mice

- Subtask 1: Examine changes in urothelial transepithelial resistance (TER) following SCI. Mice will be sacked and bladders isolated for TER and urothelial permeability measurements using Ussing chambers. **Initiated, ~10% completed.**
- Subtask 2: Histological and immunohistochemical assessment: H&E staining will be used to visually access urothelial integrity and TUNEL staining – to determine the numbers of apoptotic cells. **Completed.**

Major Task 3: Determine spinal nerve populations affected by SCI and the effect of LM11A-31/LM22B-10 on their survival.

- Subtask 1: Changes in density, localization and morphology of EUS- and bladder-labeled spinal neurons will be compared between spinal sections from control, SCI and treated animals. **Initiated, 50% completed.**
- Subtask 2: Colocalization of bladder- and EUS-labeled neurons with $p75^{NTR}$, CGRP, VIP, substance P, neurofilament-200, TRPV-1, TH and dopamine-β-hydroxylase to determine sensory and sympathetic nerve populations will be examined using immunofluorescence. **Initiated, 60% completed.**
- Subtask 3: Nissl staining will be used in spinal cord sections to determine the SCI- or treatment-induced changes in neuronal soma. **Completed**

Major Task 4: Determine the functional changes to bladder sensory nerves following SCI to address the effect of LM11A-31/LM22B-10 treatment.

- Subtask: *In vitro* single unit bladder-nerve recordings will be used to determine changes in afferent sensitivity due to SCI and treatment. **Not initiated.**

Major Task 5: Dissemination of experimental findings.

- Subtask 1: Present findings at International Continence Society and DoD-sponsored scientific meeting. **Completed**
- Subtask 2: Prepare and submit manuscripts for publication in research journals. **Initiated,** preparing manuscript for submission to Journal of Experimental Neuroscience.

What was accomplished under these goals?

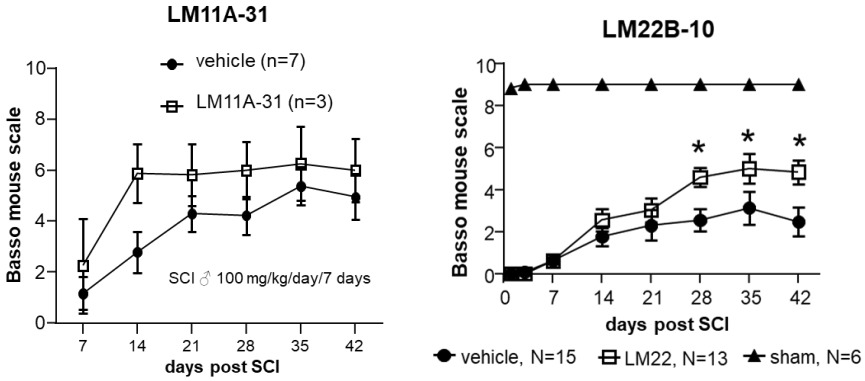
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. The project investigated the mode of action and therapeutic effect of LM11A-31 and LM22B-10 in severe SCI mouse models. Functional recovery was evaluated by comparison of lower urinary tract activity and hindlimb locomotion between treatment groups. Histological assessments of bladder and spinal cord were used to support functional data to show diminished injury to both tissues.

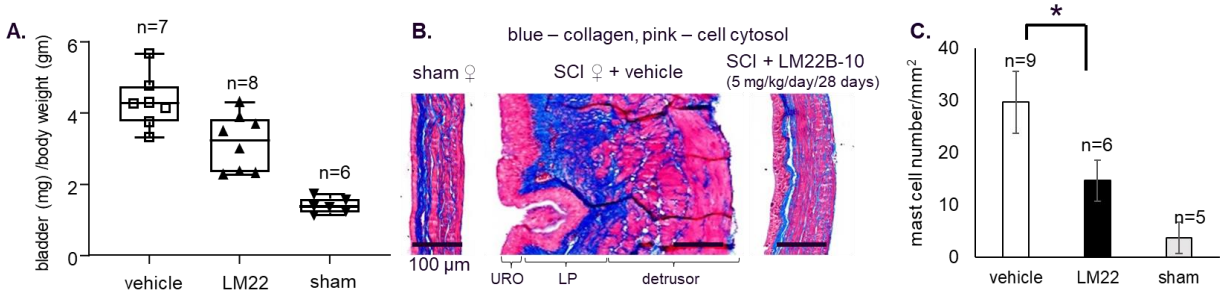
2. The objectives were to demonstrate the benefit of p75NTR modulation and TrkB/C receptor activation in functional recovery after SCI and support further investigation in translational studies.

3. The major accomplishment of this project was to determine that LM11A-31 and LM22B-10 both ameliorate secondary injury after spinal cord contusion through different mechanisms at distinct time points. LM11A-31 prevents activation of apoptotic pathways by proneurotrophin signaling that occur within 24hours of injury. LM22B-10 acts to support neural growth and survival at later stages of SCI where there is dysregulation of neurotrophin signaling. These data were reported in Continence journal, published in the inaugural issue, March 2022.

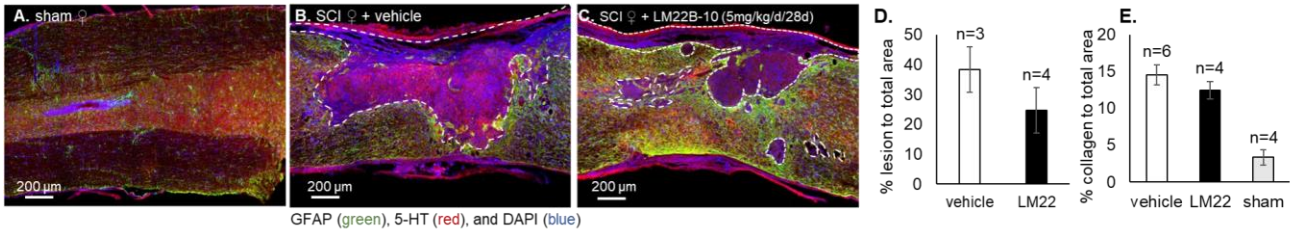
   a) LM11A-31 and LM22B-10 improve hindlimb locomotion recovery assessed using the Basso mouse scale showed maximal efficacy for LM11A-31 at 14 days and LM22B-10 at 28 days after SCI.



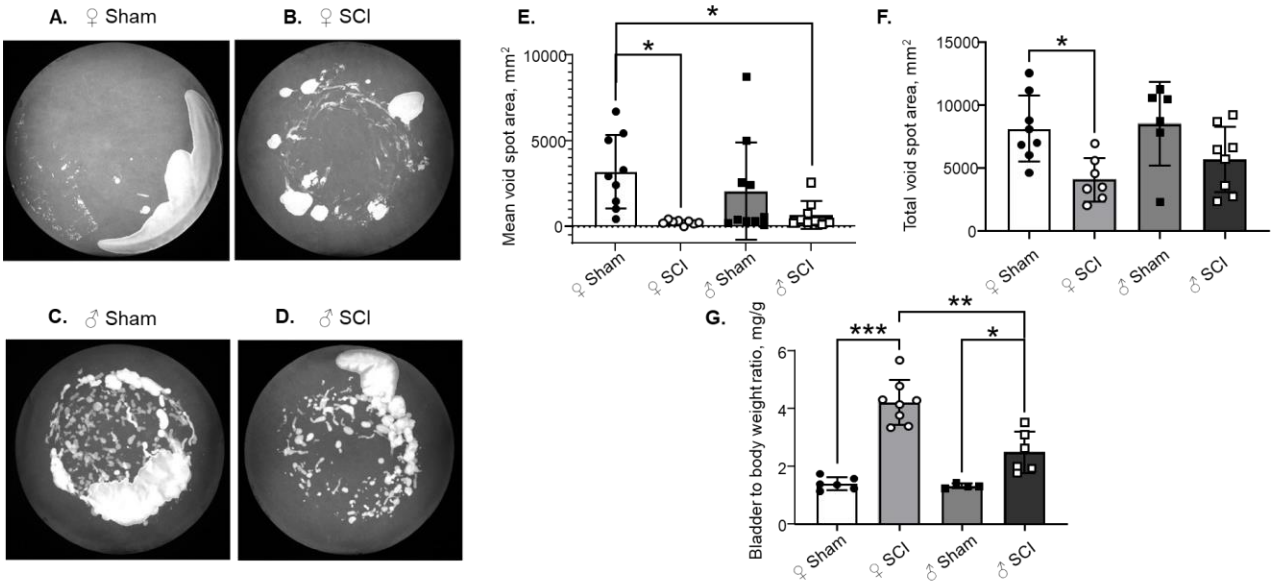
   b) LM22B-10 treatment reduced bladder hypertrophy (A&B) and mast cell infiltration (C) indicating reduced urinary retention and inflammation.



   c) LM22B-10 treatment resulted in reduced spinal cord lesion area (A-D; shown by area devoid of glial fibrillary acidic protein-GFAP) and secondary injury without affecting scar formation (D; interpreted as collagen deposition around lesion). Nissl staining and imaging of spinal cord sections were performed but still require quantification.



d) Differential effect of SCI on bladder dysfunction in male and female mice was also observed during the project. Severe contusion injury in female mice resulted in decreased urine output (A versus B) due to retention and detrusor sphincter dyssynergia which correlated with increased bladder hypertrophy (G). Male mice showed no significant difference in voiding profile and had significantly less bladder hypertrophy. These indicate that mice exhibit sex differences in bladder dysfunction after SCI.



4. The tasks that were not completed during the project include urothelial transepithelial resistance recordings, nerve track tracing *and in vitro* bladder afferent nerve recordings. We are planning to continue with the nerve track tracing studies using magnetic resonance/diffusor tensor imaging in fixed spinal cord columns instead of viral injections. There are currently no plans to continue with transepithelial resistance measurements and afferent nerve recordings.

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The key finding from the project was that there is a time-dependent effect of neurotrophins in SCI pathophysiology. Firstly, the release of proneurotrophins immediately after injury can drive cell death in the tissue surrounding the spinal cord lesion. This can be mitigated by early treatment with LM11A-31 within 24 hours of injury when urinary proneurotrophin levels are at their highest. In later stages of SCI, there can be alterations in the spinal levels of mature neurotrophins and their receptors which determine neuronal survival and growth. Administration of LM22B-10, the TrkB/C neurotrophin receptor agonist, enhanced hindlimb locomotion and bladder function at chronic stages of SCI suggesting the drug supported neural growth at this time point. These findings support the temporal changes in neurotrophin signaling following SCI and that different pharmacological interventions at key time points could mitigate secondary tissue injury and facilitate functional recovery.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

The TrkB/C receptor agonist LM22B-10 was investigated along with p75 neurotrophin receptor modulators. The rationale being that LM11A-31 acted as a protective agent during the proneurotrophin mediated apoptotic cascade in acute stages of SCI. However, it did not appear that the drug promoted significant neural regeneration or amelioration of the lesion at later stages. Thus, LM22B-10 which has shown pro-survival and growth effects in neural tissue was included to determine if in combination with LM11A-31, could enhance functional recovery.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

No significant problems were experienced during project period in the execution of experimental procedures.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

There was no significant impact of expenditures during the project period.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

No significant changes in use or care of spinal cord injured mouse model used during the project period.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  1. Ikeda Y, Zabbarova Z, Tyagi P, Hitchens TK, Wolf-Johnston A, Wipf P, Kanai A, Targeting neurotrophin and nitric oxide signaling to treat spinal cord injury and associated neurogenic bladder overactivity, Continence, Vol.1, 1000014, 2022 – https://doi.org/10.1016/j.cont.2022.100014. Published; acknowledgement – yes.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Published Conference Abstracts

1. Ikeda Y, Zabbarova I, Kozlowski M, Birder L, Kanai A, Novel telemetry recordings from the urinary bladder and external urethral sphincter in awake freely moving mice. *Neurourology and Urodynamics*, Vol 38(S3); s42-43, 2019.
2. Ikeda Y, Zabbarova I, Getchell S, Kozlowski M, Birder L, Kanai A, Selective small molecule TrkB/C agonist, LM22B-10, improves voiding function in spinal cord contused mice, *Neurourology and Urodynamics*, Vol 38(S3); s42-43, 2019.
3. Ikeda Y, Zabbarova I, Kozlowski M, Birder L, Kanai A, Diminution of the spinal cord collagen scar with concomitant improvement in gait, detrusor-sphincter-dyssynergia and bladder overactivity in spinal cord contused mice using LM22B-10, *Neurourology and Urodynamics*, Vol39(S2); S231-2, 2020.

Presentations during 2021

Ikeda Y, Zabbarova I, Kozlowski M, Kanai A, Selective small molecule TrkB/C agonist, LM22B-10, improves functional recovery of the lower urinary tract in spinal cord contused mice, Oral presentation (https://dom.pitt.edu/research-day/2021-research-day/), The Proceedings of the 17th Annual Research Day, Department of Medicine, University of Pittsburgh, 2021.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  None.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  None.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  None.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Anthony Kanai
Project Role: Principal investigator
Research Identifier: AKANAI

Name: Youko Ikeda
Project Role: Co-PI
Research Identifier: YIKEDA

Name: Irina Zabbarova
Project Role: Collaborator
Research Identifier: IZABBAROVA

Name: Mark Kozlowski
Project Role: Technician
Research Identifier: n/a

No change from previous report.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

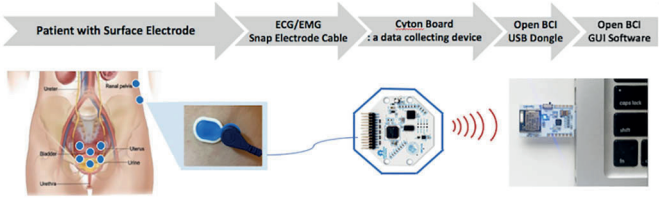
COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES:

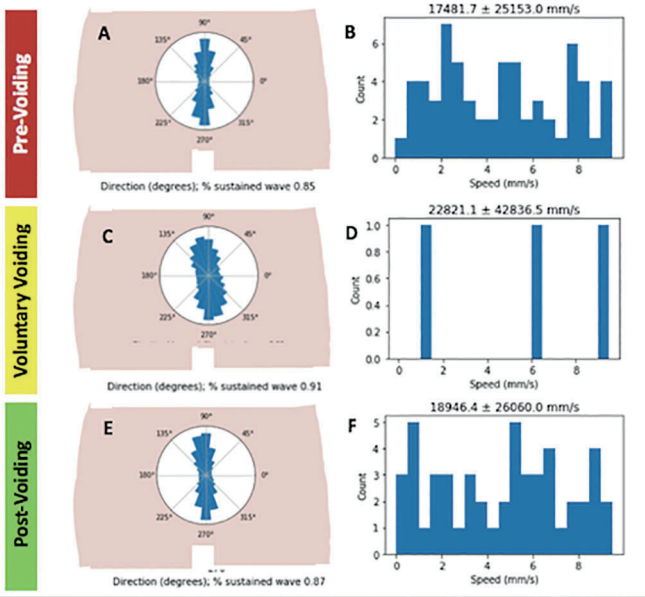
Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

FIGURE 1



Non-invasive multi-channel urodynamics (NI multi-channel urodynamics) system transferred electrical signals of the bladder from human patient utilizing ECG/EMG snap electrode cable, cyton board, Open BCI USB dongle andOPEN BCI GUI software.

FIGURE 2



Direction plot shows consistent direction of existing electrical signal in the bladder over the course of voluntary voiding (A, C, D). Speed distribution plot of electrical signal of the bladder shows that electrical activity in the bladder was almost qui

Funding One of the authors have a patent filing based upon determining spatiotemporal patterns from cutaneous multi-electrode recordings. The other authors do not have a conflict of interest. None of external funding of the study or grant to be stated for this study. **Clinical Trial** No **Subjects** Human **Ethics Committee** This study was performed in strict accordance with the recommendations in the Guide for Institutional Review Board (IRB). Data from this study does not include protected health information (PHI) of the participated subjects, therefore, a partial waiver of HIPAA authorization form was filed by all subjects in advance to the study. **Helsinki** Yes **Informed Consent** Yes

20 www.ics.org/2019/abstract/20

NOVEL TELEMETRY RECORDINGS FROM THE URINARY BLADDER AND EXTERNAL URETHRAL SPHINCTER IN AWAKE FREELY MOBILE MICE

Ikeda Y[1], Zabbarova I[1], Kozlowski M[1], Kanai A[1]
1.University of Pittsburgh

HYPOTHESIS / AIMS OF STUDY

There have been a variety of methodologies developed to obtain functional recordings from the lower urinary tract (LUT) of various animal models. These include intravesical pressure recordings from the bladder and electromyograms from the external urethral sphincter (EUS). Simultaneous recordings from both structures are essential to obtaining a clear indication of voiding and storage functions for effective comparison with clinical data. However, in order to achieve this in animal models, most methodologies use anaesthesia which dampens central nervous system (CNS) reflexes. Other methods such as whole-body restraints which are accompanied by motion artefact due to struggling and can cause stress to the animal. Decerebration cystometry does not require anaesthesia but removes CNS influence and could impact interpretation of pain-induced responses. Telemetry bladder pressure recordings have been achieved in a rat model [1] however, to our knowledge, simultaneous bladder and EUS activity by radiotelemetry has not been recorded in awake rodents. Therefore, we have developed a technique to record bladder and EUS activity in freely mobile unanaesthetised mice utilizing implantable telemeters combined with a metabolic cage system with custom load cells fitted with filter paper to measure urine void volumes, flow rates and spot patterns.

STUDY DESIGN, MATERIALS AND METHODS

Telemetry implantation: Adult (8-12 weeks) female and male C57Bl/6 mice were anesthetized with 1.5-2% isoflurane and, under sterile conditions, a lower midline incision was made to expose the urinary bladder and proximal/mid urethra. An HDX-11 telemeter (Figure 1A; Data Sciences International) was adapted to allow for implantation of a pressure catheter via the bladder dome and insertion of recording electrodes (50-micron stainless steel wire) into the EUS muscle (Figure 1B). Telemeter sending units were placed subcutaneously on the flank of the animal and mice were allowed to recover with prophylactic analgesic and antibiotics.

Metabolic cage recordings: Customized metabolic cages (Columbus Instruments) were designed to accommodate mice for up to 72-hour periods whilst recording food and water consumption in a sound-proofed, climate-controlled cabinet (Figure 1C). Each metabolic cage was equipped with a high sensitivity load cell balance set to acquire at 10 Hz frequency to record the time, volume and the speed of

voids as well as urine spot patterns. Load cells were lined with Whatman paper before animals were placed in cages to record urine spot patterns. The data from the telemeters and metabolic cage load cells were recorded using LabChart 8 (ADInstruments). Metabolic cage recordings were taken from each mouse before telemeter implantation, 1, 7, 14 and 28 days post implantation (DPI). Voided volumes (based on load cell recordings), bladder pressure, EUS-EMG recordings and time between voiding events were measured for each session. Data were expressed as mean ± standard deviation; Student's t-test was used to evaluate group differences between non-implanted and implanted data sets.

RESULTS

Bladder pressure and EUS electrical activity were recorded from both female and male mice (N=4 each) for up to three months in recording periods lasting up to six hours; telemeter batteries last up to 30 days but are only activated during recording session being switched on/off using an external magnet. Micturition contractions recorded through telemetry indicated by a sharp rise in bladder pressure, were correlated with decreased tonic firing of the EUS indicating relaxation of the sphincter muscle (Figure 2A and 2B). These events were followed by the measurement of urine flow rates and volumes, after a 0.5-1 second time delay for the urine to reach the load cells (Figure 2C). Voiding patterns were measured using filter paper mounted on the load cells and corresponded to the number of recorded voids (Figure 2D). Before telemeter implantation, female and male mice voided 263 ± 164 and 569 ± 83 µl, respectively. At 1 DPI, there was a sharp and significant decline in voided volumes in both sexes (females - 28.3 ± 12.7 µl, males - 55.8 ± 32.5 µl; $p<0.05$ versus controls without implants; Figure 2E) which were recovered by 14 DPI. The decline in voided volumes correlated with decreases in the time between voiding events (Figure 2F) which also returned to control levels by 14 DPI. There was no significant change in baseline, threshold and maximal voiding pressure values as a result of implantation surgery and between the sexes (not shown).

INTERPRETATION OF RESULTS

We have successfully recorded simultaneous bladder and EUS activity using telemetry implants in awake freely moving mice. The implantation surgery initially result in a decrease of voided volumes and increased frequency of voids, which were normalized by 14-28 DPI. The presented methodology has the added advantage of giving an accurate measurements of voided volumes, rates of voiding and voiding patterns.

CONCLUDING MESSAGE

Bladder and EUS activity can be drastically different in recordings from awake freely moving animals compared to other cytometric methodologies, making it difficult to correlate findings from animal studies to the human situation. This may be particularly important in studying neurogenic bladder dysfunction which often is associated with discoordination of the bladder and urethral activity. Our telemetry technique has the potential for long-term monitoring of LUT function in animal models to better characterize pathologies and determine the efficacy of treatments.

FIGURE 1

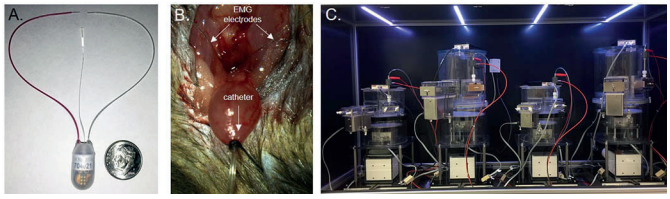


Figure 1. Images of the HDX-11 telemeter, implanted catheter and EMG electrodes and metabolic cage setup.

FIGURE 2

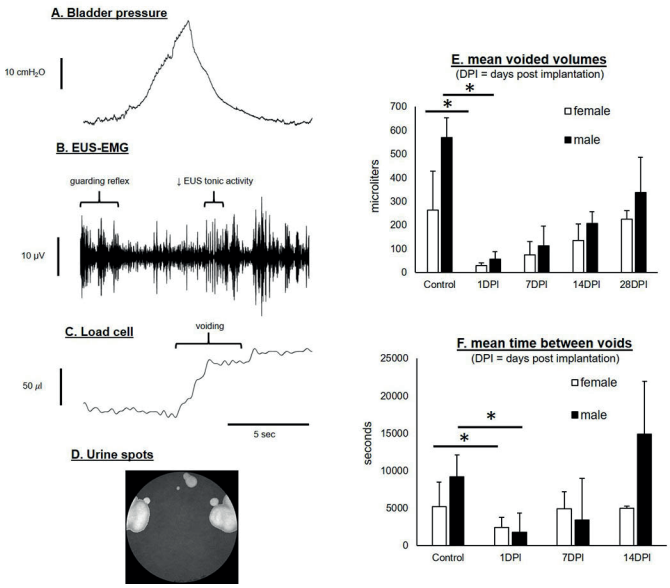


Figure 2. Example telemetry recordings of A) bladder pressure, B) EUS-EMG, C) load cell and resulting D) urine spots from a male mouse. E) change in voided volume and F) time between voids following telemeter implantation.

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Funding Awards from NIH/NIDDK; R01 DK071085 (Kanai), R01 DK098361 (Kanai and Drake), P01 DK093424 (Kanai) and Department of defense SC170171 (Kanai and Ikeda) **Clinical Trial** No **Subjects** Animal **Species** mouse **Ethics Committee** University of Pittsburgh Institutional Animal Care and Use Committee

count of c-Fos (+) cells/ 10 sections was 11.3 ± 2.3, 17.5 ± 6.2, 12.6 ± 1.9, 12.8 ± 5.2, and 15.3 ± 1.2 in medial dorsal horn; 14 ± 2.2, 17.5 ± 3.5, 15.8 ± 2.0, 8.6 ± 1.2, and 6.7 ± 0.7 in lateral dorsal horn; 78 ± 22, 219 ± 26, 203 ± 21, 318 ± 24, and 155 ± 1.7 in dorsal commissure; 26 ± 5.6, 96 ± 24, 89 ± 19, 90 ± 7.5, and 69 ± 11 in sacral parasympathetic nucleus (control vs. fast infusion vs. LPS vs. ATP vs. AA group, respectively, means ± SEM). Both mechanical stimuli by fast infusion and chemical stimuli by LPS, ATP, and AA instillation were revealed to induce the c-Fos (+) cells in dorsal commissure and sacral parasympathetic nucleus of the L6-S1 spinal cord (figure 2).

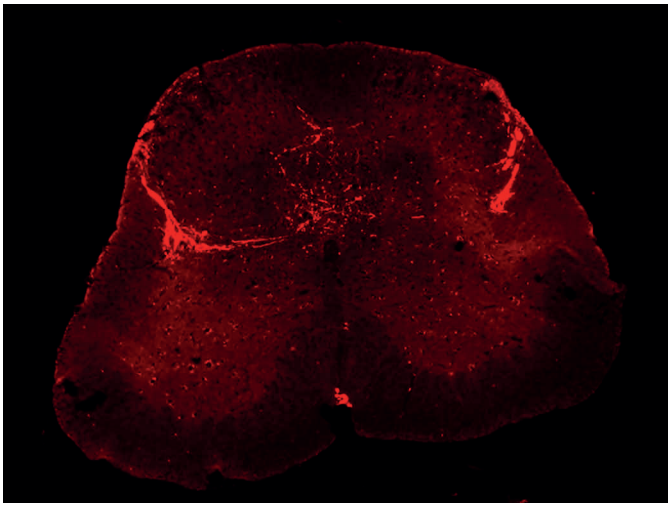
INTERPRETATION OF RESULTS

These findings suggest that both mechanical and chemical stimuli conveyed from the bladder are processed by the spinal neurons of dorsal commissure and sacral parasympathetic nucleus but not of medial dorsal horn and lateral dorsal horn in mice. This is not consistent with that of rats and cats, because noxious stimuli has been reported to increase the number of c-Fos (+) cells in medial dorsal horn, sacral parasympathetic nucleus, and dorsal commissure in these animals. Therefore, the neural control system for voiding reflex of mice must be different from that of rats and cats.

CONCLUDING MESSAGE

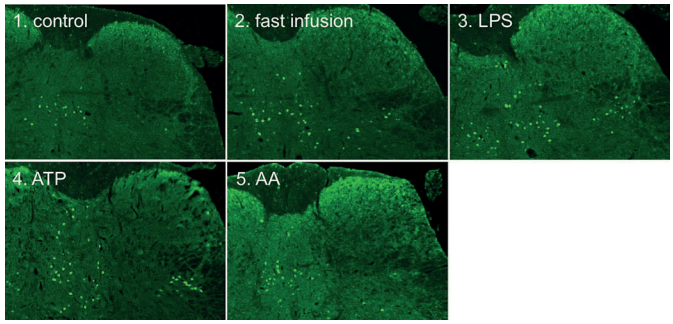
Mouse bladder primary afferent pathways are suggested to project to the neurons of dorsal commissure and sacral parasympathetic nucleus but not of medial dorsal horn and lateral dorsal horn in L6-S1 spinal cord. These findings are different from those of rats and cats, and suggest different neural control system for voiding reflex between mice and these animals.

FIGURE 1



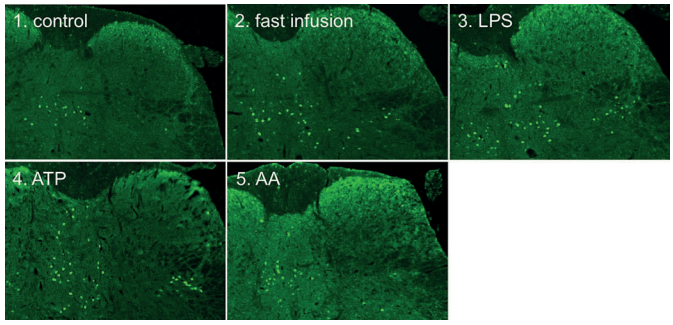
distribution pattern of Cholera toxin B

FIGURE 2



spinal c-fos induction caused by various bladder stimuli

FIGURE 3



spinal c-fos induction caused by various bladder stimuli

Funding non **Clinical Trial** No **Subjects** Animal **Species** Mouse **Ethics Committee** Institutional Animal Care and Use Committee of Osaka University

211

www.ics.org/2019/abstract/211

SELECTIVE SMALL MOLECULE TRKB/C AGONIST, LM22B-10, IMPROVES VOIDING FUNCTION IN SPINAL CORD CONTUSED MICE

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HYPOTHESIS / AIMS OF STUDY

Spinal cord injury (SCI) can have detrimental consequences to the lower urinary tract (LUT) resulting in loss of voluntary bladder control, decreased bladder compliance, increased risk of infections and potentially damage to the upper urinary tract. Neurotrophins have been implicated to play a significant role in the development of neurogenic bladder overactivity (NDO), where nerve growth factor (NGF) has been implicated in the sensitization of C-fibre afferent pathways and detrusor overactivity following SCI. Interestingly, brain derived neurotrophic factor (BDNF) also plays a crucial role in the development of SCI-induced LUT dysfunction by counteracting spinal neural remodelling and inhibiting the

emergence of NDO [1]. Therefore, our aim was to determine the therapeutic potential of LM22B-10, a selective small molecule agonist of TrkB/C receptors, in ameliorating voiding dysfunction using a mouse model of incomplete spinal cord injury.

STUDY DESIGN, MATERIALS AND METHODS

Spinal cord contusion surgery and functional assessments: Female C57Bl/6 mice were anesthetized using 1.5-2% isoflurane, a laminectomy performed and the spinal cord exposed between T9-T10 vertebrae. The exposed cord was subjected to severe contusion injury (75 kDy force; Infinite Horizon Impactor, Precision Instrument). Sham controls underwent laminectomy surgeries without contusion. The surgical wound was packed with haemostatic sponge and the muscle and skin sutured. After surgery, the animals had their bladders expressed twice a day by gentle abdominal compression and given daily prophylactic antibiotics and analgesics for up to one week. Mice were evaluated for hindlimb locomotion recovery using the Basso mouse scale [2] and two-hour urine spot tests at one, three, seven, 14, 21 and 28 days following injury. The effects of LM22B-10 were evaluated by subcutaneous implantation of osmotic pumps to deliver 5 mg/kg/day of drug or vehicle over a two-week period; pumps were implanted at 21 to 28 days post-SCI surgeries.

Immunofluorescence: Mice were anesthetized with 5% isoflurane, the urinary bladder dissected out and the mouse was transcardially perfused with 1 x tris-buffered saline (1xTBS) followed by 4% paraformaldehyde for dissection of the spinal cord. Bladders were fixed flat in a dissection dish with 4% PFA. Spinal cord and bladder tissues were stored overnight in 30% sucrose solution then embedded in optimal cutting medium for cryosectioning. Tissue section were washed in 1xTBS, blocked with 10% normal donkey serum + 0.1% Triton-X in 1xTBS and incubated overnight in primary antibodies at 4°C. Sections were then incubated in appropriate fluorescent secondary antibodies and visualized using an Olympus IX62 fluorescent upright microscope.

RESULTS

Following SCI, urine spot tests showed that injured mice exhibited decreased voided volume and increased urinary frequency (presented as multiple small urine spots) compared to sham controls (Figure 1A versus 1B). A two-week treatment with LM22B-10 normalized the voiding pattern of SCI mice to that comparable with sham operated mice (Figure 1C versus 1A). There did not appear to be a significant improvement in the hindlimb locomotion recovery in LM22B-10 treated mice compared to vehicle treated group (not shown). Chronic SCI resulted in the extensive deposition of collagen fibers at the site of injury (Gomori's trichrome staining, Figure 2A-C). Expression of TrkB receptors was increased in the spinal cord around the injury site and appeared to be predominantly associated with growth associated protein-43 (GAP-43) or glial fibrillary acidic protein (GFAP; not shown) positive cells surrounding the fibrotic glial scar (Figure 2B, D and F). SCI-induced upregulation of TrkB receptors in the spinal cord was ameliorated by LM22B-10 treatment (Figure 2F versus 2D).

INTERPRETATION OF RESULTS

These data demonstrate activation of TrkB/C signalling by LM22B-10 has a positive effect in ameliorating SCI-induced bladder overactivity in the mouse model. Our data showed contusion SCI resulted in increased TrkB expression at the site of injury that appeared to be associated with astrocytes or glial cells. Astrocytes selectively express a truncated TrkB isoform that when activated induces their proliferation, release of inflammatory cytokines and formation on the glial scar [3]; thus, hindering neural regeneration and in turn recovery of LUT function. It is hypothesized that LM22B-10 may elicits its beneficial effects by downregulating truncated TrkB expression on astrocytes and/or biased activation of regenerative signalling pathways.

CONCLUDING MESSAGE

Our data demonstrate that a two-week administration of the TrkB/C agonist, LM22B-10, improved voiding function in chronic SCI mice that was associated with decreased expression of TrkB at the injury site. Modulation of neurotrophin signalling with small molecules could represent a novel treatment for SCI-induced LUT dysfunction.

FIGURE 1

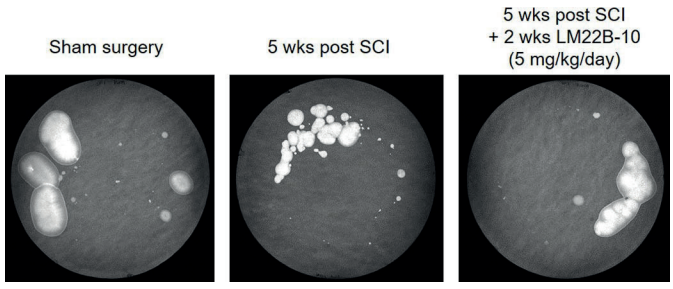

Figure 1. Urine spot tests from sham control and spinal cord injured mice with and without LM22B-10 treatment.

FIGURE 2

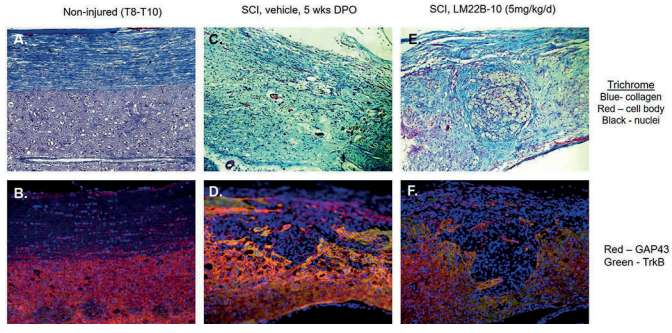

Figure 2. Histological and immunofluorescence images of T8-T10 spinal cord from control and spinal cord injured mice with and without LM22B-10 treatment.

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2. Basso et al., J Neurotrauma, 23(5):635-59, 2006
3. Matyas J et al., J Neursci., 37(14):3956-3971, 2017

Funding Awards from NIH/NIDDK; DK098361 (Kanai and Drake), P01 DK093424 (Kanai) and Department of defense SC170171 (Kanai and Ikeda) **Clinical Trial** No **Subjects** Animal **Species** mouse **Ethics Committee** University of Pittsburgh Institutional Animal Care and Use Committee

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NERVE GROWTH FACTOR-MEDIATED NA+ CHANNEL PLASTICITY OF BLADDER AFFERENT NEURONS IN MICE WITH SPINAL CORD INJURY

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HYPOTHESIS / AIMS OF STUDY
Lower urinary tract dysfunction (LUTD) is a common complication of spinal cord injury (SCI) and a major cause of death. It has been reported that nerve growth factor (NGF) is a crucial mediator involved in the emergence of LUTD after SCI. Previous studies demonstrated that neutralization of NGF can reduce non-voiding contractions (NVCs), improve voiding efficiency and restore the hyperexcitability of bladder afferent neurons in SCI mice. Na+ current is a dominant factor of neuronal excitability. There are nine subtypes of Nav channels found throughout the body (Nav1.1-1.9), and Nav 1.6-1.9 channels were detected in isolated muscle afferent neurons. Previous studies demonstrated that Nav channels existed in bladder afferent neurons and a transition from tetrodotoxin(TTX)-resistant to TTX-sensitive of Na+ current was found after SCI. Here, we aim to investigate the effect of NGF neutralization on Na+ channel plasticity of bladder afferent neurons in mice with spinal cord injury.

STUDY DESIGN, MATERIALS AND METHODS
Thirty-sixth female C57/BL6 mice were randomly divided into three groups: spinally intact (SI) group, SCI group and SCI+NGF Ab group. SCI was conducted by transection at the Th8/9 level. In SCI+NGF Ab group, anti-NGF antibodies (10 μg•kg-1 per hour) were administered subcutaneously for 2 weeks by osmotic pump. Bladder was emptied by pressing twice a day for SCI mice. Bladder afferent neurons were labelled with Fluoro-gold (FG), injected into the bladder wall three weeks after SCI. Four weeks after SCI, The cystometrograms (CMGs) of all group were recorded to evaluate the effect of NGF on bladder function. Besides, L6-S1 dorsal root ganglion (DRG) neurons were dissociated and whole cell patch clamp recordings were performed on FG-labelled neurons. Action potential (AP) and Na+ current were recorded before and after TTX intervention. L6-S1 DRGs were harvested for immunofluorescence (IF) staining of Nav1.7 and Nav1.8 subtypes.

RESULTS
The results of CMG showed the bladder overactivity, performed as increased non-voiding contractions (NVCs), in SCI mice. NGF neutralizing treatment could significantly reduce the number of NVCs and improve the voiding efficiency. The whole-cell patch clamp recordings showed that the excitability of bladder afferent DRG neurons was significantly increased in SCI mice, performed as lower threshold of AP and multiple firing pattern. Besides, TTX could partly inhibit AP and Na+ current of bladder afferent neuron in SI mice, which are almost completely inhibited in SCI mice. (Figure 1) Specifically, the data showed increased total Na+ current, TTX-Sensitive Na+ current and decreased TTX-Resistant Na+ current after SCI. (Figure 2) These changes of physiological properties can be partially reversed by NGF-antibody treatment. IF staining showed that Nav1.7 was significantly increased and Nav1.8 was significantly decreased in bladder afferent neurons after SCI, which can be restored by NGF-antibody treatment.

INTERPRETATION OF RESULTS
These results indicate that (1) Increased level of NGF can induce bladder overactivity after SCI, and NGF neutralizing treatment is effective for restoring the bladder function; (2) SCI can induce a transition of Na+ channels from TTX-resistant to TTX-sensitive subtypes, which is associated with the increased level of NGF and can be restored by NGF neutralizing treatment. (3) SCI can induce increased expression of Nav1.7 (TTX-sensitive) and decreased expression of Nav1.8 (TTX-resistant), which can be restored by NGF neutralizing treatment. Thus, it is assumed that increased level of NGF after SCI can mediate Na+ channel plasticity of bladder afferent neurons that TTX-resistant subtypes shift to TTX-sensitive subtypes, and result in bladder overactivity.

CONCLUDING MESSAGE
Foregoing results indicate that NGF mediates the Na+ channel plasticity that TTX-resistant subtypes (Nav 1.8) changed to TTX-sensitive subtypes (Nav 1.7), and this might be an underlying mechanism of bladder overactivity after SCI. Besides, NGF, Nav1.7 and Nav1.8 subtypes therapies could be effective for treatment of neurogenic bladder dysfunction after SCI.

SESSION 19 (PODIUM) - BEST BASIC SCIENCE
Abstracts 253-258
09:30 - 11:00, Brasilia 4
Chairs: Prof Christopher Henry Fry (United Kingdom), Dr Toby C. Chai (United States)

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BEST NON-CLINICAL ABSTRACT

DIMINUTION OF THE SPINAL CORD COLLAGEN SCAR WITH CONCOMITANT IMPROVEMENT IN GAIT, DETRUSOR-SPHINCTER-DYSSYNERGIA AND BLADDER OVERACTIVITY IN SPINAL CORD CONTUSED MICE USING LM22B-10

Ikeda Y[1], Zabbarova I[1], Kozlowski M[1], Birder L[1], Kanai A[1]
1. University of Pittsburgh

HYPOTHESIS / AIMS OF STUDY

Spinal cord injury (SCI), besides resulting in paralysis, can induce lower urinary tract (LUT) dysfunctions that are also highly debilitating and increase morbidity in afflicted individuals. Neurogenic LUT dysfunction includes bladder overactivity, detrusor-sphincter dyssynergia (DSD), urinary retention, frequent urinary tract infections and potential damage to the upper urinary tract. Alterations in neurotrophin signalling has been shown to play a major role in the development of LUT dysfunction. Previous studies have indicated a time dependent downregulation in brain derived neurotrophic factor (BDNF) dependent signalling that results in further deterioration of LUT function [1]. Compensating for the decrease in BDNF may represent a treatment for preventing degeneration of LUT function. Thus, our aim was to elucidate whether LM22B-10, a selective small molecule agonist of TrkB/C receptors [2], affects the development of bladder overactivity and DSD in a spinal cord contused mouse model.

STUDY DESIGN, MATERIALS AND METHODS

Spinal cord contusion (SCC) surgery and functional assessments: Adult female C57Bl/6 mice (8-12 weeks old) were anesthetized using 2% isoflurane, a laminectomy performed, and the spinal cord exposed between T9-T10 vertebrae. The exposed cord was subjected to severe contusion injury (75 kDy force; Infinite Horizon Impactor, Precision Instrument). Sham controls underwent laminectomy surgeries without contusion (N=4). The area partially devoid of column segments was packed with haemostatic sponge and the muscle and skin sutured. After surgery, the animals had their bladders expressed twice daily by gentle abdominal compression and were given daily prophylactic antibiotics and analgesics for up to one week. Mice were evaluated for hindlimb locomotion recovery using the Basso mouse scale [3] at one, three, seven, 14, 28 and 42-days following injury. LM22B-10 was administered by subcutaneous implanted osmotic pumps that delivered 5 mg/kg/day of drug (N=8) or vehicle (N=9) over a four-week period (Alzet model 1004, vehicle consisted of 50% DMSO with sterile saline). Osmotic pumps were implanted at the time of SCC surgeries without priming which delays drug release by 24-48 hours. At 42 days post SCC, mice were subjected to decerebrate cystometrogram and external urethral sphincter electromyogram (CMG-EUS-EMG) recordings to examine bladder and EUS activities, respectively.

Histology: Following cystometric measurements, the urinary bladder was dissected out and the mouse transcardially perfused with 1 x tris-buffered saline (1xTBS) followed by 4% paraformaldehyde for perfusion fixation of the spinal cord and bladder. Bladders were weighed then further fixed flat in a dissection dish with 4% PFA. Spinal cord and bladder tissues were stored overnight in 30% sucrose solution then embedded in optimal cutting temperature compound for cryosectioning. Spinal cord and bladder tissue sections were processed for immunofluorescence to image glial fibrillary acidic protein (GFAP) and TrkB receptors and/or to Trichome staining for visualization of tissue collagen content. Slides were imaged using brightfield montage microscopy (Olympus Fluoview3000) and analyzed using HCImage software (Hamamatsu Photonics). Quantitative data were expressed as mean ± SEM. Unpaired Student's t-test determined differences between contused vs. sham controls and parameters with and without treatment. One-way ANOVA multiple comparison was performed to determine between group differences followed by Tukey's multiple comparisons test.

RESULTS

Histological evaluation of spinal cord injury sites showed reduced scarring and decreased collagen deposition (Fig. 1A, white circles indicate necrotic core) and by void area bound by GFAP and TrkB expressing cells in the spinal cords (Fig. 1B) of LM22B-10 treated mice compared to vehicle controls. Furthermore, LM22B-10 treated mice showed improvement in hindlimb locomotion recovery at 28- and 42-days post injury compared to vehicle treated group (Fig. 1C). Bladders of LM22B-10 treated SCC mice also showed decreased collagen deposition and bladder hypertrophy (Fig. 1E and 1F) compared to controls. Continuous filling CMG recordings in chronic SCC mice demonstrated bladder overactivity and non-voiding contractions that were not present in sham controls (Fig. 2B vs. 2A). EUS-EMG recordings from sham mice show a guarding reflex as bladder pressure increased, and decreased EUS tonic activity accompanied by bursting as the bladder emptied; characteristic of normal rodent voiding (Fig. 2A inset, tonic EUS activity denoted by red arrows). Conversely, vehicle treated SCC mice showed high

baseline pressures, consistent non-voiding contractions with increased EUS tonic activity during bladder contraction which is the hallmark of DSD. (Fig 2B inset). SCC mice that received LM22B-10 treatment exhibited reduced numbers of non-voiding contractions that correlated decreased tonic activity (Fig. 2C) when compared to vehicle controls.

INTERPRETATION OF RESULTS

These data demonstrate that activation of TrkB/C signalling by LM22B-10 reduced bladder overactivity and DSD in the contused mouse model. Our data showed that contusion injury resulted in the formation of large scar regions and secondary cysts within the spinal cord that can prevent neural regeneration. This scar region was smaller in LM22B-10 treated mice which suggests that the drug may be eliciting its positive effects by reducing deposition of inhibitory extracellular proteins around the glial scar and/or promoting neural growth.

CONCLUDING MESSAGE

LM22B-10 treatment improved hindlimb function and reduced the development of bladder overactivity and DSD in chronic SCC mice that correlated with decreased spinal cord scar volume and secondary injuries. Targeting neurotrophin signalling pathways could hold significant potential as a treatment for neurogenic LUT dysfunction.

FIGURE 1

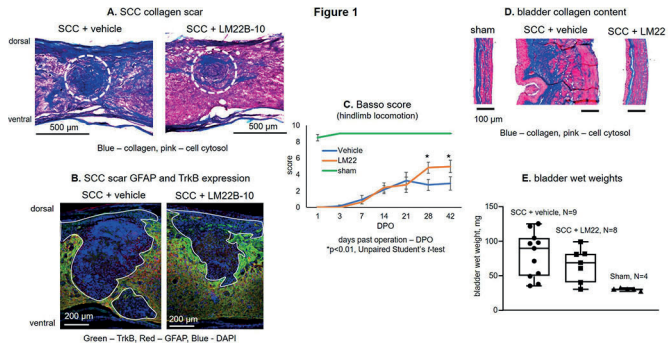
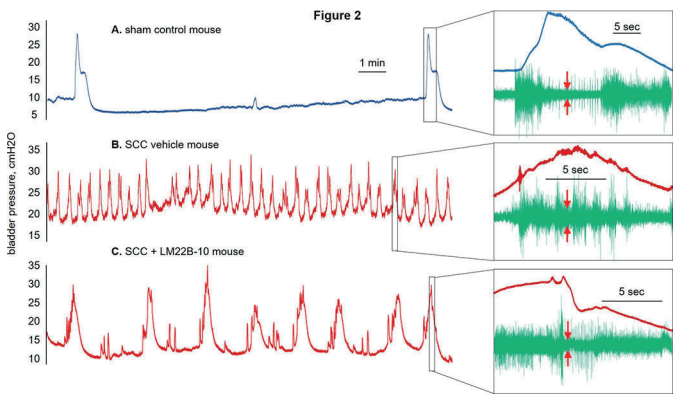


FIGURE 2



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3. Basso et al., J Neurotrauma, 23(5):635-59, 2006

Funding Department of Defense **Clinical Trial** No **Subjects** Animal **Species** Mouse **Ethics Committee** Institutional Animal Care and Use Committee

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BEST IN CATEGORY PRIZE "CONTINENCE CARE PRODUCTS / DEVICES / TECHNOLOGIES"

MEASUREMENT OF FELINE BLADDER PRESSURE AND VOLUME USING CATHETER-FREE WIRELESS INTRAVESICAL SENSOR

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1. Louis Stokes Cleveland VA Medical Center, 2. Cleveland Clinic Lerner Research Institute

HYPOTHESIS / AIMS OF STUDY

Lower urinary tract neurophysiology research relies on animal models (e.g. feline) with implanted nerve recording and stimulating electrodes. These systems enable conscious recording of peripheral nerve data, e.g. from the dorsal root ganglia [1], but there are few options for simultaneous measurements of bladder function. In research animal models, catheterization for cystometry requires anesthesia which affects neuro-urological pathways, or animal restraint, limiting measurement time and social behaviors surrounding natural bladder filling and micturition. To overcome this limitation, we developed a catheter-free, wireless, intravesical sensor that is implanted into the bladder lumen of felines and is designed to transmit untethered bladder pressure and volume data in the absence of a wire crossing the detrusor tethering the bladder [2]. This research tool is expected to enable studies in physiologically-relevant settings, and would allow long-term monitoring of lower urinary tract changes in reaction to neuromodulation or pharmacologic interventions. This study validated sensor function and physiologic outcomes over 4-week implantations, including untethered catheter-free wireless recordings of bladder pressure during natural bladder filling and voiding in felines.

STUDY DESIGN, MATERIALS AND METHODS

A wireless sensor incorporating low-power pressure-sensing electronics, platinum electrodes for measuring urine concentration and conductance for volume estimation, and antennas for wireless battery recharge and data transmission was developed [3]. The sensor transmitted data 10 times per second to an external antenna up to 20 cm away. The external antenna was connected to a pager-like wearable radio for ambulatory data recording. Conscious wireless recharge

Continence 1 (2022) 100014

Contents lists available at ScienceDirect

Continence

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Targeting neurotrophin and nitric oxide signaling to treat spinal cord injury and associated neurogenic bladder overactivity

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[b] University of Pittsburgh, School of Medicine, Department of Pharmacology & Chemical Biology, USA
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ARTICLE INFO

Keywords:
Detrusor sphincter dyssynergia (DSD)
Neurogenic detrusor overactivity (NDO)
p75 neurotrophin receptor ($p75^{NTR}$)
Soluble guanylate cyclase (sGC) activators
Spinal cord injury/contusion (SCI/SCC)
TrkB/C receptors

ABSTRACT

Purpose or the research: Nearly 300,000 people are affected by spinal cord injury (SCI) with approximately 18,000 new cases annually, according to the National SCI Statistics Center. SCI affects physical mobility and impairs the function of multiple internal organs to cause lower urinary tract (LUT) dysfunctions manifesting as detrusor sphincter dyssynergia (DSD) and neurogenic detrusor overactivity (NDO) with detrimental consequences to the quality of life and increased morbidity. Multiple lines of evidence now support time dependent evolution of the complex SCI pathology which requires a multipronged treatment approach of immediate, specialized care after spinal cord trauma bookended by physical rehabilitation to improve the clinical outcomes. Instead of one size fits all treatment approach, we propose adaptive drug treatment to counter the time dependent evolution of SCI pathology, with three small molecule drugs with distinctive sites of action for the recovery of multiple functions.

Principal results: Our findings demonstrate the improvement in the recovery of hindlimb mobility and bladder function of spinal cord contused mice following administration of small molecules targeting neurotrophin receptors, LM11A-31 and LM22B-10. While LM11A-31 reduced the cell death in the spinal cord, LM22B-10 promoted cell survival and axonal growth. Moreover, the soluble guanylate cyclase (sGC) activator, cinaciguat, enhanced the revascularization of the SCI injury site to promote vessel formation, dilation, and increased perfusion.

Major conclusions: Our adaptive three drug cocktail targets different stages of SCI and LUTD pathology: neuroprotective effect of LM11A-31 retards the cell death that occurs in the early stages of SCI; and LM22B-10 and cinaciguat promote neural remodeling and reperfusion at later stages to repair spinal cord scarring, DSD and NDO. LM11A-31 and cinaciguat have passed phase I and IIa clinical trials and possess significant potential for accelerated clinical testing in SCI/LUTD patients.

1. Introduction

Neurogenic injury can have severe consequences to multiple physiological functions including mobility and autonomic regulation of internal organs. Lower urinary tract dysfunction (LUTD) is especially prevalent in neurological injury/disease. There are several pharmacological and surgical interventions available that can alleviate the consequences of LUTD, however, they do not restore complete function. Thus, there is still a necessity to expand the knowledge on LUT pathology and identify additional therapeutic targets to improve the functional outcomes. Thus, we posited that multiple, synergistic interventions are required to achieve the highest possible therapeutic results.

Neurotrophins are a family of growth factors that include nerve growth factor (NGF), brain derived neurotrophic factor (BNDF) and neurotrophin-3 and -4 (NT-3/4) (Table 1) that have been attributed to play a key role in the development of LUTD after SCI and modulation of their activity has shown to ameliorate aspects of bladder and urethral sphincter dysfunctions [1,2]. Neurotrophins elicit their actions through the tropomyosin-related kinase (Trk) receptors that are known to activate various cell survival and growth pathways (Fig. 1A).

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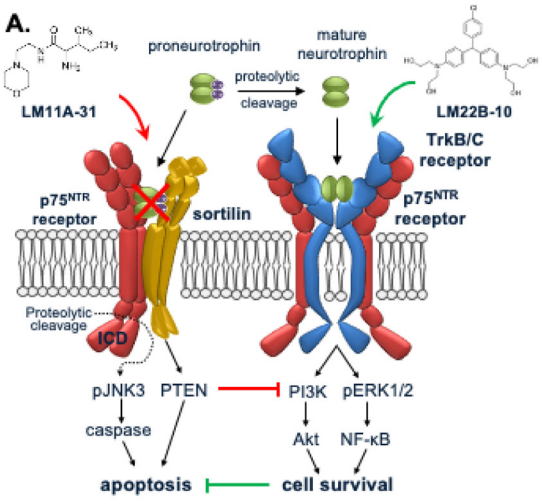

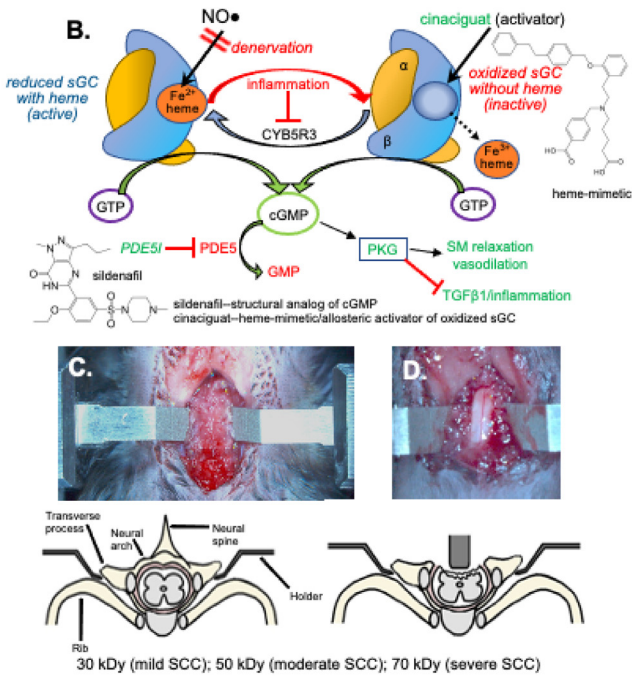

Table 1. neurotrophin receptors and known ligands

Receptor	Ligand
$p75^{NTR}$	pro-NGF, -BDNF, -NT3, -NT4
TrkA	NGF
TrkB	BDNF/NT-4
TrkC	NT-3

Fig. 1. $p75^{NTR}$ *and sGC-cGMP signaling pathways*. A. While the $p75^{NTR}$-sortilin complex preferentially binds with proNGF/proBDNF to activate apoptotic signaling cascades and $p75^{NTR}$-Trk complex binds with mature neurotrophins to activate cell survival. LM11A-31 targets the $p75^{NTR}$-sortilin complex to prevent the activation of apoptotic cascades. Table 1. Neurotrophin receptors and their ligands. B. Since cGMP production by NO• requires a reduced heme (Fe^{2+}) in the active site of sGC, reduction of sGC heme by CYB5R3 sensitizes it to NO•. The unresponsiveness of heme free sGC to NO• in pathology is reverted by sGC activators are heme mimetics, which do not require NO• or heme to stimulate cGMP production. Images of male mouse T_8–T_9 spinal cord segments C. before and D. after laminectomy. Fig. 1A and Table 1 are adapted from reference [6] and Fig. 1B is adapted from I. Zabbarova et al., J Pathol. 2022 Apr; 256(4):442–454.

NGF has been primarily associated with the remodeling of sensory innervation and development of the bladder to spinal cord reflex that induces detrusor contraction in response to distention [3]. BNDF has also been implicated in the reflex activity and development of DSD [4]. Previous studies have also implicated the precursor neurotrophins, proneurotrophins, as the initiator of bladder injury following SCI by inducing urothelial layer disruption and apoptosis through its action on the p75 neurotrophin receptor ($p75^{NTR}$, Fig. 1A) [5,6]. Accordingly, the therapeutic potential of neurotrophin receptors has led to the identification of LM11A-31 and LM22B-10, small molecule modulators of $p75^{NTR}$ and Trk B/C receptors, respectively [7–10]. LM11A-31 selectively acts on $p75^{NTR}$ to inhibit its interaction with proneurotrophins which are elevated in neurodegenerative conditions such as SCI [5,6] and Alzheimer's disease [8,10], thus, LM11A-31 can act as an anti-apoptotic agent. LM22B-10 is a full agonist of TrkB/C receptors and demonstrated to stimulate neural survival and growth in the brain [7].

Vascular dysfunction is also associated with SCI and bladder overactivity [11] which is believed to occur in response to inflammation, oxidative stress and endothelial damage. A hallmark of vascular damage is the impaired responsiveness to nitric oxide (NO•), a key mediator of vascular smooth muscle relaxation. NO• activates soluble guanylate cyclase (sGC) to generate cyclic guanosine monophosphate (cGMP) that stimulates a multitude of downstream pathways. There are indications that increasing NO•-cGMP signaling can be beneficial in ameliorating SCI induced detrusor overactivity [12]. However, approved agents such as phosphodiesterase (PDE) type 5 inhibitors may have limited efficacy due to decreased NO• bioavailability and/or oxidative inhibition of sGC, essentially preventing cGMP generation. Small molecule sGC activators could circumvent these pathological changes as they can allosterically activate sGC in the absence of its heme prosthetic group and NO• (Fig. 1B) [13]. Studies in SCI rat bladder strips indicate there is decreased responsiveness to NO• donors and PDE5 inhibitors [14], suggestive of sGC dysfunction.

Here we describe investigations of small molecule neurotrophin receptor modulators and a sGC activator to enhance neural sparing and ameliorate LUTD after thoracic level spinal cord contusion injury using a mouse model. The rationale for use of multiple agents is that there are distinct phases in the pathological progression after neurogenic injury and differential treatment is required at each stage. For example, proneurotrophin levels surge immediately following SCI peaking within 24 h [5,6] and trigger apoptotic cascades that have detrimental long-term consequences. Thus, it is postulated that administration of LM11A-31 within this period may prevent these adverse outcomes. Our objective was to determine their potential as therapeutic agents to promote the recovery of mobility and bladder function after SCI.

2. Materials and methods

2.1. Animals

Adult female and male C57Bl/6 mice (8–12 weeks) were purchased from Envigo (Indianapolis, IN) and maintained in a centralized climate-controlled animal facility on a 12 h light/dark cycle (7am to 7pm). Mice were socially housed (four to five mice per cage) in microisolator caging units where food and water were provided ad libitum. Ethical approval was obtained from the Institutional Animal Care and Use Committee for all animal work described.

2.2. Spinal cord contusion (SCC) injury

Mice were anesthetized with isoflurane (5% induction, 2.5% maintenance in 100% O_2) and a laminectomy was performed under sterile surgical conditions between T_8–T_{10} vertebrae to expose the dorsal spinal cord for contusion injury. The musculature around the vertebrae was bluntly dissected and the vertebrae column was secured using a customized holder before removal of the lamina. The exposed cord was subjected to a severe contusion injury (Fig. 1C–D, 75 kilodyne) using the IH-0400 Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC). Immediately following contusion, the exposed cord was covered with hemostatic sponge and in a select cohort, an

osmotic pump (ALZET #1004) with LM22B-10 (5 mg/kg/day, Tocris, Minneapolis, MN) or vehicle was placed subcutaneously on the flank of the mouse. The muscle and skin layers were sutured separately, and recovery observed for two hours after surgery. Mice were monitored twice daily during the first week after surgery, provided prophylactic antibiotic, ampicillin (100 mg/kg twice daily for seven days) and analgesic, ketoprofen (3 mg/kg, twice daily for three days) and manual bladder expression performed until reflex voiding was established (approximately 5 to 7 days after injury). Sham animals were subjected to the laminectomy surgery without contusion injury.

2.3. Hindlimb locomotion assessments

Recovery of hindlimb function was assessed at one, three, seven, 14, 28, 35 and 42 days following contusion using the Basso Mouse Scale (BMS) method [15] based on observations of leg movement, weight bearing, plantar stepping motion, coordination and trunk stability. Mice were evaluated using the BMS scoring sheet by at least two assessors at each time point with evaluation performed independently. The score sheet was used to derive the primary BMS score (0–9) and a sub-score (0–11) which was used to further refine differences in stepping activity.

2.4. Drugs

Cinaciguat (Tocris, Minneapolis, MN) was dissolved in DMSO, suspended in 1% methylcellulose and administered by daily gavage (10 mg/kg). LM11A-31 (synthesized in house) was dissolved in sterile water and administered by daily gavage (100 mg/kg). LM22B-10 (Tocris) was dissolved in 50% DMSO and 50% sterile saline and filled into osmotic pumps under aseptic conditions.

2.5. Fluorescent bead vascular labeling

Mice were anesthetized with isoflurane and a PE10 catheter inserted into the jugular vein for perfusion with 1xPBS with 500 U heparin using a syringe pump at 10 μl/min followed by solution with fluorescent microbeads (F8787, Thermofisher, 0.02 μm) for 10–15 min. Animals were sacrificed immediately after perfusion, their spinal cords removed and fixed in 4% paraformaldehyde (PFA) for 48 h followed by incubation in graded sucrose. Tissues were frozen in optimal cutting temperature medium and sectioned 100–400 μm thick on a cryostat. Sections were cleared using the ClearT2 method [16], mounted onto slides, and imaged using confocal microscopy (FV3000, Olympus, Tokyo, JP).

2.6. Mitochondrial respiration assessment

Mitochondria were isolated from control, SCC and SCC treated with cinaciguat C57Bl/6 mouse spinal cords as described previously [17] with modifications. After deep anesthesia with isoflurane (2%–5%), the spinal cord (≈60 mg) was isolated by extrusion [18] and placed in a mitochondrial solution containing 5 mM HEPES, 125 mM KCl, 2 mM monobasic KH_2PO_4, 20 μM EDTA, 5 mM $MgCl_2$ and 0.2 mg/ml BSA, adjusted to pH 7.4 with 100 mM KOH. The spinal cord was minced by a McIlwain motorized tissue chopper (Brinkmann) set to chop at a 10 μm interval, placed in 10 ml of mitochondrial solution and homogenized by a few passes with a motorized (75 rpm) Teflon pestle. The homogenate was spun at 1000 × g for 10 min. The supernatant was again spun at 10,000 × g for 10 min to obtain a second pellet containing the mitochondria. Pellets were resuspended in 100 μl of mitochondrial solution, and 25 μl of the suspension placed in a gas-tight vessel containing a Clark-type oxygen microelectrode (MI-730/OM-4; Microelectrodes, Londonderry, NH) to measure the state 3 (succinate + ADP) and state 4 (succinate alone) respiratory rates. The electrode was calibrated considering the total amount of dissolved O_2 in aqueous solution after equilibration with air at 36 °C to be 215 μM [19] and zeroed with sodium dithionite. The respiratory control ratio (RCR), a measure of the "tightness of coupling" between electron transport and oxidative phosphorylation, was determined from the ratio of state 3 to state 4 rates of respiration. A RCR of 2–4 is considered good for complex II substrates [20].

2.7. Magnetic resonance imaging (MRI)/Diffusion tensor imaging (DTI) of isolated spinal cords

PFA fixed mouse spinal cords were dissected to 20 mm long lumbar–cervical segments and placed in a 5 mm glass NMR tube containing perfluorocarbon oil (Fluorinert FC-40, Sigma-Aldrich, St Louis, MO) as a magnetic susceptibility matching fluid. A glass Shigemi NMR tube plunger was inserted to secure the tissue in place. MRI was performed using a Bruker AV3HD 11.7 Tesla/89 mm vertical-bore microimaging system equipped with a 16-channel shim insert and Micro2.5 gradient set with a maximum gradient of 1500 mT/m and data collected using a 5 mm RF resonator and ParaVision 6.0.1 (Bruker Biospin, Billerical MA). Sample temperature was maintained at 22 °C using a variable temperature module. Following pilot scans, the field homogeneity was optimized with field map shimming. Anatomical MRI scans were acquired using a three dimensional T2-weighted RARE TR/TE 1000/36 ms, 15.6 × 5 × 5 mm FOV at 100 mm isotropic resolution. Diffusion Tensor Imaging were collected with a spin-echo sequence TR/TE 2000/26 ms, with Stejskal–Tanner diffusion scheme with D/d = 15/6 ms, two shells (b=1000, 2000 s/mm ^{2}), a total of 18 diffusion encoding directions with two B0 images, fifty 0.3 mm slices, 5 mm FOV, 64 × 64 matrix and 8 averages. Diffusion tensor images were reconstructed with DSI Studio software. Regions of Interest (ROIs) were manually drawn in the white matter on each image slice and plots of DTI scalar measures, Fractional Anisotropy (FA), Mean Diffusivity (MD), Axial Diffusivity (AD) and Radial Diffusivity (RD), will be generated vs. slice position.

2.8. Histology and immunofluorescent analysis

At the experimental endpoints, mice were placed under deep isoflurane anesthesia and a thoracotomy was performed. The mouse was immediately transcardially perfused with 1x tris buffered saline (TBS) followed by 10% neutral buffered formalin (NBF). Spinal cord segments for histology were isolated and placed in 10% NBF overnight followed by 30% sucrose for cryopreservation. Segments were sectioned 20 μm thick on a cryostat and stored at −30 °C until processed for immunofluorescence or staining. Collagen content in the spinal lesion was assessed by Van Gieson staining (MilliporeSigma, Burlington, MA) with omission of hematoxylin/elastin stain. Immunofluorescent detection was performed as previously described [21] and antibodies utilized are summarized Table A.1 in the Appendix. Collagen content and spinal cord lesion area quantification was performed with FIJI ImageJ software and averaged from three sequential sections for each animal.

2.9. Statistical analysis

Continuous measures were expressed as mean ± standard error of the mean (SEM). Between group differences were assessed by one-way ANOVA with Tukey's post-hoc multiple comparison analysis (Prism 9, GraphPad) and significance was determined as $p<0.05$.

3. Theory

We hypothesize that temporal changes in the levels of paracrine signaling messengers at the injury site in the spinal cord drive the initiation and the spread of neurodegeneration following SCI. There is an initial surge in the proneurotrophins that are released almost immediately after the injury to activate apoptotic signaling. Proneurotrophins levels start to decrease within 24 h after which there is partial recovery. However, altered neurotrophin activity over time

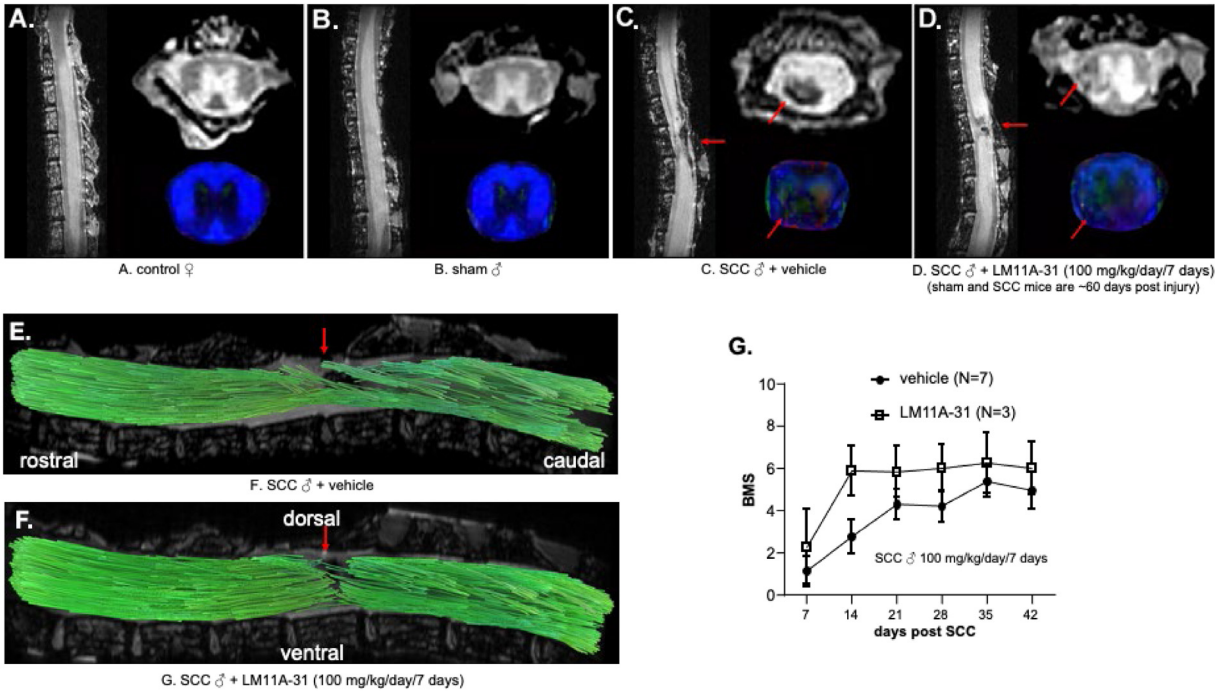

Fig. 2. *Magnetic resonance imaging (MRI) of spinal cords and plots of diffusion tensor imaging (DTI) vs. position.* Images from: A. control, B. sham, C. contused, and D. LM11A-31 treated mice. The panels display T_2-weighted images in the sagittal plane (left), axial plane (top right), and directionally encoded color (DEC) maps, bottom right. MR images are grayscale and the hues in DEC maps index the orientation of fractional anisotropy (FA). For intact cords (panels A–B), anterior/posterior FA is displayed by blue; for injured cords (C–D), changes in FA are displayed by green (dorsal/ventral) and red (left/right) at the lesion (red arrows). The integrity of the gray matter (central region of the cord) in D compared to C demonstrates that LM11A-31 limits tissue degeneration. DTI data is from 15 mm fields of view (FOV) in 0.3 mm slices centered on the injury site. Fiber tracking of E. vehicle and F. LM11A-31 treated contused mouse cords using data from C and D, respectively; the red arrows to the sites of injury. G. Basso mouse score (BMS) for male SCC mice following daily gavage with LM11A-31 (100 mg/kg) or vehicle.

is further compounded by the overproduction of cerebrospinal fluid which hydrostatically opposes neovascularization and the resulting deprivation of oxygen at the injury site inhibits neuroregeneration. Our proposed adaptive multipronged action of three drugs is predicted on time dependent changes in paracrine signaling for achieving synergistic action on multiple signaling mechanisms to support functional recovery and tissue repair. We propose targeting three distinct receptor pathways; the $p75^{NTR}$, TrkB/C and NO•-cGMP pathways using novel pharmacological agents to inhibit neuronal and urothelial apoptosis, to promote growth/survival and to support angiogenesis/repair for improved functional recovery.

4. Results

4.1. LM11A-31 promotes functional recovery by sparing the nerve fiber tracks visualized with MRI and diffusion tensor imaging (DTI)

DTI (Fig. 2) relies on the orientation of directional diffusion of water molecules within the tissues. Inside the spinal cord, water diffusion in white matter axons is limited by the cell membrane and diffusion mainly occurs parallel to the white matter tracts with less diffusion in the perpendicular direction. SCI damages the cell membrane to cause unrestricted perpendicular water diffusion radial to the site of injury [22]. Accordingly, DTI allows the visualization of nerve tracts over the entire spinal cord in a more time efficient and accurate manner. DTI was performed on the thoracic region of non-injured (Fig. 2A), sham operated male (Fig. 2B), SCC male (Fig. 2C) and SCC male mice given LM11A-31 for the first 7 days after surgery, starting within three hours after surgery (Fig. 2D). Tissues were collected 60 days after surgery. These studies demonstrate the feasibility of MRI to distinguish the injury and repair of white matter tracts in the mouse spinal cord for discerning the neuroprotection by LM11A-31 which engenders greater tissue sparing compared to vehicle SCC mice, as shown by MR images, diffusivity measurements and fiber tracking (Fig. 2E and F). Hindlimb locomotion assessments correlated with MR imaging showing significant improvement with LM11A-31 compared to the vehicle treated mice at 14 days post SCI (Fig. 2G).

4.2. LM22B-10 improves the recovery of hindlimb function and ameliorates neurogenic bladder dysfunction

Female C57Bl/6 mice were subjected to severe contusion injury (Fig. 1C–D) and at the time of injury also implanted with osmotic pumps to deliver the TrkB/C selective agonist, LM22B-10 (5 mg/kg/day) [7] or vehicle over a 4-week period. Assessment of hindlimb movement showed that the LM22B-10 treated cohort had significantly better hindlimb recovery at 28, 35 and 42 days after surgery (Fig. 3A–B). Recovery of hindlimb movement correlated with an improved LUT function as demonstrated in decerebrate filling cystometrogram (CMG) and EUS electromyogram (EMG) recordings (Fig. 3C–E). LM22B-10 treated SCC mice had less frequent non-voiding contractions (NVCs) and exhibition of DSD, and improved voiding efficiency ($n \geq 4$). The decreased time windows to the right of the traces demonstrate the increase in tonic activity of the EUS in SCC animal (Fig. 3D) indicative of DSD. The EMG trace in Fig. 3E demonstrates that the EUS tonic activity decreases as does the bladder pressure indicative of a small void occurring.

4.3. LM22B-10 decreases the spinal cord lesion area but not collagen content

To assess whether LM22B-10 was promoting recovery *via* enhanced survival of neural tissue, we examined histological sections of spinal cord segments around the lesion. Longitudinal sections (rostral to caudal) were obtained and the localization of glial fibrillary acidic

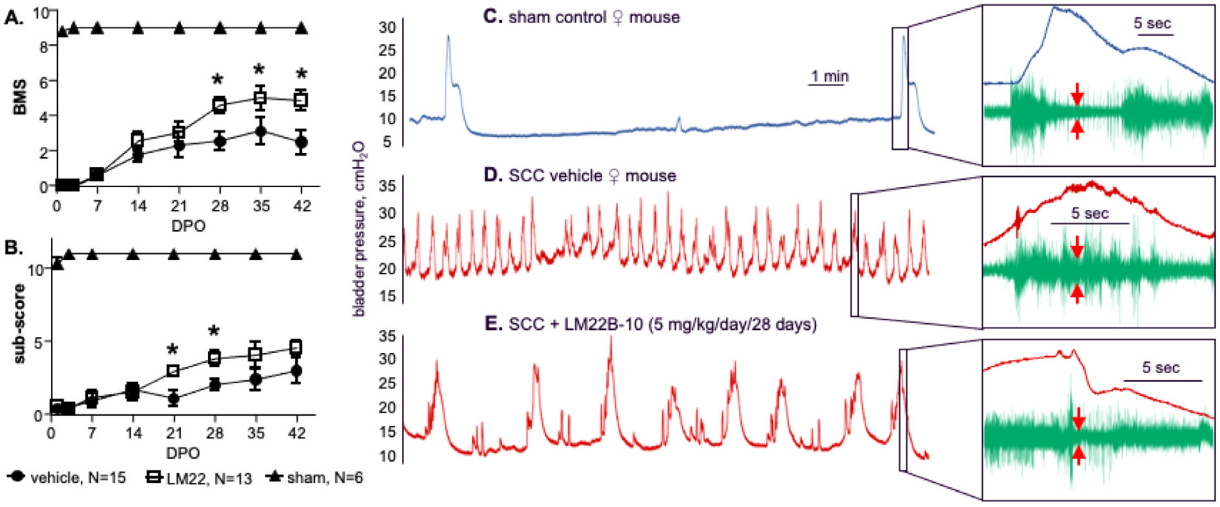

Fig. 3. *Improved hindlimb locomotion recovery and LUT function in LM22B-10 treated SCC mice.* A. Basso hindlimb locomotion scores and B. sub-scores from SCC with vehicle, SCC with LM22B-10 treatment and sham female mice between 3 to 42 days post operation (DPO). Scoring was performed by two trained assessors blinded to the test group designation independently. Mean ±SEM, *= $p<0.05$, LM22B-10 *vs.* vehicle SCC, one-way ANOVA with Tukey's multiple comparison test. Decerebrate CMG–EUS–EMG recordings from C. sham female, D. SCC with vehicle and E. SCC with LM22B-10 treatment. Breakout boxes show expanded time bases with EUS-EMG traces demonstrating changes in tonic firing during bladder contractions. Reduction in EUS tonic firing during bladder contraction facilitates urine outflow.

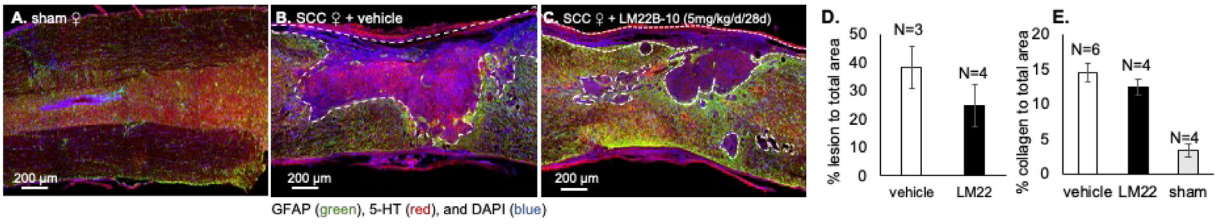

Fig. 4. *LM22B-10 reduces total spinal cord lesion area but not collagen deposition.* Immunofluorescent image of T_8–T_9 spinal cord section labeled with glial fibrillary acidic protein (GFAP, green), 5-hydroxytryptophan (5-HT, red) and DAPI (blue) from A. a sham female mouse B. SCC female mouse with vehicle treatment. C. SCC female mouse treated with LM22B-10 (5 mg/kg/day/28days). D. Bar chart of % lesion area *vs.* total measurement area, determined from an area 2 mm on either side of the lesion epicenter. E. Bar chart of % collagen in T_8–T_9 spinal cord sections from SCC vehicle, SCC LM22B-10 treated and sham female mice. Comparable sections were processed with Van Gieson stain omitting hematoxylin. Percentage area of collagen (not shown) was calculated *vs.* total measured area.

protein (GFAP, marker for activated astrocytes/glia—green) and 5-hydroxytrypamine (5-HT, transmitter in interneurons—red) was examined by immunofluorescence (Fig. 4A–C). Chronic SCC resulted in a significant disruption of the spinal cord architecture (Fig. 4B). In SCC mice, a large lesion extended along the dorsal aspect, delineated by GFAP positive cells. LM22B-10 treated SCC mice showed a reduction in the overall lesion area and frequently exhibited smaller lesions scattered around the injury epicenter (Fig. 4C). Comparable sections examined with Van Gieson stain without hematoxylin revealed that collagen infiltration at the spinal cord scar was not significantly different between vehicle and LM22B-10 cohorts (Fig. 4D–E).

4.4. Cinaciguat improves mitochondrial respiration in SCC mice

The neuro protective effect of cinaciguat in the spinal cord was assessed by measuring the respiration of mitochondria (Fig. 5) isolated from spinal cords of female sham, SCC, and SCC mice given cinaciguat (10 mg/kg/day/7days). These experiments showed that a decrease in RCR following SCC was normalized by cinaciguat (Table 2). Improved RCR values with cinaciguat treatment correlated with the high density of blood vessels around the spinal cord lesion (Fig. 5D–F).

5. Discussion

These findings provide empirical support for targeting three distinct signaling pathways using novel pharmacological agents to inhibit apoptosis and promote growth/survival while also supporting angiogenesis/repair at different time points following SCI. We evaluated morphological changes in the spinal cord using MRI, microbead infusion with confocal imaging and histological techniques, and assessed functional recovery of urinary bladder activity using cystometry and hindlimb locomotion. These assessments were supported by molecular techniques including protein analysis for biomarkers and mitochondrial respiration measurements of spinal cord tissues.

LM11A-31 has been shown to selectively bind with $p75^{NTR}$ to inhibit proneurotrophin induced apoptosis [8]. We have previously reported on the efficacy of LM11A-31 on the amelioration of LUTD in SCI mice with complete spinal cord transections [5,6] where it is thought to act on the urothelium and nerves innervating the urinary bladder. In this study, we demonstrated administration of LM11A-31 within three hours after SCI and continued daily for seven days resulted in improved hindlimb recovery and nerve track sparing (Fig. 2). Irrespective of the spinal level of injury, SCI leads to rewiring within the lumbosacral spinal cord to establish a reflex between bladder sensory nerves and preganglionic parasympathetic nuclei and/or EUS motoneurons [3]. This is largely driven by the initial surge of proneurotrophins in the bladder which disrupts urothelial integrity, initiates neural degeneration and promotes inflammation to cause LUTD. Since the abrupt rise in proneurotrophins subsides quickly after peaking within 24 h after SCI [6], we reasoned that the administration of LM11A-31 should occur as soon as possible after stabilization of the patient as a prophylactic intervention for slowing down the neurodegeneration by limiting the proneurotrophin induced apoptotic cascade.

In silico analysis of LM11A-31 congeners for affinity to neurotrophin receptors [7] led to the identification of LM22B-10 as a TrkB/C selective agonist for promoting the survival and growth of hippocampal

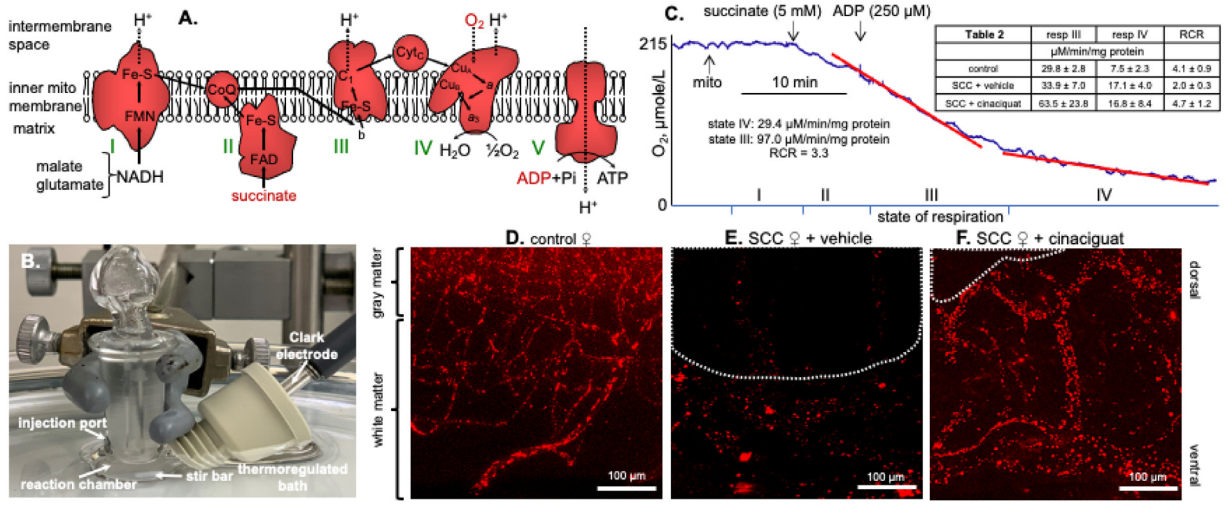

Fig. 5. *Spinal cord mitochondrial respiration and blood vessel density.* A. The five mitochondrial complexes located near the inner membrane. B. The glass chamber used for mitochondrial respiration measurements positioned in a thermoregulated water bath C. Typical oxygen consumption measurement by a Clark microelectrode depicting the four states of mitochondrial respiration: I, respiration with internal substrates; II, respiration stimulated by additional substrates (succinate for complex II); III, the state induced by addition of high (100–300 µM) concentrations of ADP; state IV is obtained when ADP is depleted by phosphorylation to ATP. The respiratory control ratio (RCR) is a measure of the tightness in mitochondrial coupling which is determined from the ratio of state 3 to state 4 respiration. A RCR of 3 and above with succinate as substrate is considered tightly coupled, while 1–2 indicates damaged mitochondria. Table 2. Respiration rates and RCRs of spinal cord mitochondria isolated from control, contused, and contused mice treated with cinaciguat (10 mg/kg/day/7 days). D. Confocal image of a longitudinal spinal cord section at the level of injury epicenter from a control female mouse following I.V. infusion of 0.02 µm red fluorescent microbeads. E. SCC female gavaged daily with methylcellulose (N=2). White dotted line delineates the lesion area. F. SCC female given daily cinaciguat (10 mg/kg/day, N=2).

neurons [9]. Here, we demonstrate that LM22B-10 improved mobility and ameliorated LUTD in SCI mice when given up to 28 days after injury. Our findings are supported by a previous report using the partial TrkB/C agonist, LM22A-4, which also reduced the secondary tissue injury in SCI models [23]. It is possible that further improvement could have been achieved if treatment was continued past 28 days, which would require further investigation. We propose LM22B-10 could be used as a trophic support agent for early to chronic stages of SCI. Furthermore, we did not observe increased signs of pain or distress with LM22B-10 treatment as there was no significant difference in post-surgical weight loss compared to vehicle cohort up to 42 days after SCI (not shown). Therefore, we do not anticipate LM22B-10 treatment would aggravate neuropathic pain, a potential complication with SCI.

Cinaciguat is a potent sGC activator with vasodilatory effects and was initially investigated as a treatment for acute heart failure [24]. The unique feature of sGC activators is their ability to induce cGMP generation when the sGC heme moiety is oxidized or absent and the NO•-signaling pathway is compromised. The binding of sGC heme is regulated by its redox state and only attaches when the heme is in the reduced state. This is especially important in SCI which is accompanied by systemic inflammation and chronic oxidative stress that would promote heme oxidation and inactivation of sGC. Our data show SCC resulted in significant alterations in mitochondrial RCR in spinal cord tissue which was normalized by the treatment with cinaciguat. Our data suggest that angiogenesis around the spinal cord lesion occurs slowly, leading to chronic ischemia and cellular metabolic dysfunction. sGC activators such as cinaciguat can promote angiogenesis [25] which in this case is functionally reflected as improved RCR values. Further, we propose that the efficacy of cinaciguat would be highest in damaged tissues where sGC heme oxidation/loss occur. This is supported by the limited effect of the PDE5 inhibitor, sildenafil on the spinal cord revascularization and functional recovery after SCI [26], presumably due to oxidative stress induced sGC inactivation.

A major focus of this study was to support the translational potential of the proposed drugs for enhancing the functional recovery following SCI. There are currently no drugs approved for this purpose, likely due to the complexity of SCI pathology that progresses over time and therefore not amenable to a one drug approach. Thus, we reasoned that repair of the injured spinal cord and functional recovery requires time-bound targeting of multiple signaling pathways at critical time points. The proposed route and dosing for each of the drugs were based upon their absorption, distribution, metabolism, and excretion profiles. LM11A-31 is a water-soluble drug, oral dosing of 50 mg/kg by gavage achieved peak brain concentration in mice within approximately 30 min and its uptake into the CNS is proposed to involve active transport [10]. LM22B-10 and cinaciguat are poorly water-soluble drugs that were solvated in DMSO and administered by an osmotic pump or gavage. All three drugs are excreted by hepatic clearance in feces.

6. Conclusion

Our data support there are multiple pathways that can be manipulated to improve functional outcomes following neurogenic injury. Further investigations are required into combination treatments that provide neurotrophic support and revascularization with the aim to improve recovery of mobility and bladder functions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix

(See Table A.1.)

Table A.1
List of antibodies.

Target	Dilution	Host	Manufacturer/lot number
glial fibrillary acidic protein (GFAP)	1:1,000	Rat	Thermofisher,
5-HT	1:1,000	Rabbit	Immunostar
anti-rat alexfluor-488	1:500	Donkey	Thermofisher
anti-rabbit alexfluor-594	1:500	Donkey	Thermofisher

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