Journal of Visualized Experiments

Activity-Based Training on a Treadmill with Spinal Cord Injured Wistar Rats

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58983R1
Full Title:	Activity-Based Training on a Treadmill with Spinal Cord Injured Wistar Rats
Keywords:	Spinal Cord Injury; Activity-based training; Locomotor Training; Rehabilitation; Urology; Exercise Therapy
Corresponding Author:	Charles Hubscher University of Louisville Louisville, KY UNITED STATES
Corresponding Author's Institution:	University of Louisville
Corresponding Author E-Mail:	chhubs01@exchange.louisville.edu
Order of Authors:	Jason H Gumbel
	Casey J Steadman
	Robert F Hoey
	James E Armstrong
	Jason D Fell
	Cui Bo Yang
	Lynnette R. Montgomery
	Charles Hubscher
Additional Information:	
Question	**Response**
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Ron Myers, Ph.D.
Science editor
JOVE

October 2, 2018

Dear Dr. Myers,

Attached is a PDF document of our revised manuscript entitled, “Activity-Based Training on a Treadmill with Spinal Cord Injured Wistar Rats,” which includes two tables, five figures and a Table of Materials.

Please consider our paper for publication in JOVE.

On behalf of all authors, we thank you in advance,

Charles H. Hubscher
Professor and Vice Chair
Anatomical Sciences and Neurobiology
University of Louisville
511 South Floyd Street
Louisville, KY 40202
Tel. 502-852-3058
Email: Charles.Hubscher@louisville.edu

TITLE:

Activity-based Training on a Treadmill with Spinal Cord Injured Wistar Rats

AUTHORS AND AFFILIATIONS:

Jason H. Gumbel[1], Casey J. Steadman[1], Robert F. Hoey[1], James E. Armstrong[1], Jason D. Fell[1], Cui Bo Yang[1], Lynnette R. Montgomery[1], Charles H. Hubscher[1,2]

[1]Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY, USA

[2]Kentucky Spinal Cord Injury Rehabilitation Center, University of Louisville, Louisville, KY, USA

Corresponding Author:

Charles H. Hubscher (chhubs01@louisville.edu)

Email Addresses of Co-authors:

Jason H. Gumbel (jason.gumbel@louisville.edu)
Casey J. Steadman (casey.steadman@louisville)
Robert F. Hoey (robert.hoey@louisville.edu)
James E. Armstrong (jimmy.armstrong@louisville.edu)
Jason D. Fell (Jason.fell@louisville.edu)
Cui Bo Yang (cuibo.yang@louisville.edu)
Lynnette R. Montgomery (lynnette.montgomery@louisville.edu)

KEYWORDS:

Spinal cord injury, activity-based training, locomotor training, neurology, rehabilitation, exercise therapy

SUMMARY:

This protocol demonstrates our model of activity-based locomotor treadmill training for rats with spinal cord injury (SCI). Included is both quadrupedal and forelimb-only groups, in addition to two distinct types of non-trained control groups. Investigators are able to assess training effects on SCI rats using this protocol.

ABSTRACT:

Spinal cord injury (SCI) results in lasting deficits that include both mobility and a multitude of autonomic-related dysfunctions. Locomotor training (LT) on a treadmill is widely used as a rehabilitation tool in the SCI population with many benefits and improvements to daily life. We utilize this method of activity-based task-specific training (ABT) in rodents after SCI to both elucidate the mechanisms behind such improvements and to enhance and improve upon existing clinical rehabilitation protocols. Our current goal is to determine the mechanisms underlying ABT-induced improvements in urinary, bowel, and sexual function in SCI rats after a moderate to severe level of contusion. After securing each individual animal in a custom-made adjustable vest, they are secured to a versatile body weight support mechanism, lowered to a modified three-lane treadmill and assisted in step-training for 58 minutes, once a day for 10 weeks. This setup

allows for the training of both quadrupedal and forelimb-only animals, alongside two different non-trained groups. Quadrupedal-trained animals with body weight support are aided by a technician present to assist in stepping with proper hind limb placement as necessary, while forelimb-only trained animals are raised at the caudal end to ensure no hind limb contact with the treadmill and no weight-bearing. One non-trained SCI group of animals is placed in a harness and rests next to the treadmill, while the other control SCI group remains in its home cage in the training room nearby. This paradigm allows for the training of multiple SCI animals at once, thus making it more time-efficient in addition to ensuring that our pre-clinical animal model mimics the clinical representation as close as possible, particularly with respect to the body weight support with manual assistance.

INTRODUCTION:

Globally, between 250,000 - 500,000 new spinal cord injury (SCI) cases arise either due to degeneration, diseases, or most commonly (up to 90%) trauma[1]. After traumatic SCI, a series of physiologic events take place that result in neurological deficits that affect a multitude of bodily functions. Due to the chronic deficits that follow SCI, the development and testing of effective treatment modalities is crucial. Until recently, rehabilitation strategies have most commonly focused on recovery of mobility[2,3]. Following SCI, patients rank bladder/urinary, bowel, and sexual functions among the highest quality of life complications in need of better management[1,4,5]. Therefore, targeting bladder, bowel, and sexual function is of utmost importance from a rehabilitation standpoint[1,4,5].

Exercise and locomotor training (LT) are commonly utilized rehabilitative therapies in the SCI patient population with many benefits such as cardiovascular function, bladder/urinary function, and mobility[6-10]. It is for this reason we utilize a similar modality in our pre-clinical rat SCI model. It is our goal to determine what effects LT has on SCI Wistar rats, specifically regarding both upper (kidney) and lower (bladder, external urethral sphincter) urinary tract function, bowel function, and sexual function. Further, LT has been shown to be sufficient in activating neuromuscular systems below the level of injury which may influence the amount of plasticity within the central nervous system (CNS)[11,12].

The success of LT in pre-clinical studies is well documented in both large[13,14] and small[15-19] SCI animal models. Evidence suggests that afferent sensory input provided by LT is sufficient to stimulate spinal reflex pathways that result in plasticity and improvements to sensory-motor function[9,20]. LT benefits regarding autonomic functions have not been well characterized. For this reason, we implement our training paradigm with a focus on autonomic outcome measures, using four distinct groups that include two non-trained controls and a metabolic/exercise non-weight bearing group alongside an LT group that mimics the timing, session duration, manual assistance and weight support that are used in clinical studies[19,21-24].

PROTOCOL:

All methods described have been approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC).

1. Pre-injury Handling and Testing (One Week Before SCI)

1.1 Handle each rat for a period of 5 - 10 min once a day for five days.

NOTE: Adult male Wistar rats that are ~50 days of age initially and weigh 200 - 225 g are used in this protocol. Rats at this pre-injury time-point are not acclimated to the harness that is used for LT as full use of hindlimbs allows the rat to escape from the jacket.

1.2 Conduct any pre-injury testing that is study-specific (*e.g.*, the authors do metabolic cage assessments for studies that involve the effects of SCI on bladder and bowel function).

2. Spinal Cord Contusion[25-28]

2.1 Anesthetize animals with ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture intraperitoneally according to provided dosage chart (**Table 1**). Administer supplemental dosing as needed. Test anesthetic depth at least every 10 min by assessing corneal, palpebral, pedal, tail pinch, and pinna reflexes.

2.2 Shave hair from the back of the animal where incision and injury are to occur. Administer a long-acting general antibiotic (*e.g.*, 0.5 cc Pro-Pen-G subcutaneously).

2.3 Place the anesthetized animal on a heating pad at a low setting to maintain normal body temperature.

2.4 Estimate location of targeted lesion level based upon vertebral protuberances and with a #10 scalpel, make an estimated 5 cm incision on the dorsum of the animal, directly above the midline vertebrae.

2.5 For mid-thoracic contusions, expose the T8/T9 level of spinal cord *via* removal (with rongeurs) of the overlaying T7 vertebral lamina.

2.6 Using a contusion device such as an infinite horizon impactor[29], perform the contusion (for a moderate to severe degree of SCI, use a force of 210 kdyn with no dwell time)[18].

2.7 Suture together the muscular layer and fascia over the spinal cord using 4-0 diameter monofilament and close the skin with 9 mm surgical wound clips.

2.8 Administer postoperative drugs, such as gentamicin sulfate (5 mg/kg per day for 5 days; antibiotic to avoid bladder infections) and meloxicam (1 mg/kg subcutaneously, analgesic for first 48 h and then as needed).

2.9 Place animals in a clean cage placed on heating pad and monitor closely for the first 24-48 h.

2.10 Perform bladder emptying procedures using the manual Credé maneuver 3 times a day (8 am, 3 pm, 10 pm) until reflexive bladder function has returned (3 - 6 days on average for contusions)[26,30].

3. Training Phase

3.1 Commence LT no earlier than two weeks post-SCI, as initiating interventions too early may exacerbate secondary injury cascades[31].

3.2 Week 1 acclimation to treadmill training: Transport the rats to a quiet room that is dedicated for training.

3.3 On Day 1, randomly and evenly divide the SCI animals into trained and non-trained control groups, to account for potential variability in both the injury itself as well as the degree of spontaneous recovery after contusion. For example, divide rats into 4 separate groups: quadrupedal trained (QT), forelimb-only trained (FT), non-trained control (NT), and non-trained home cage control (HC). A sham group where animals receive a laminectomy but no injury and are otherwise handled the same as the other groups can also be used as an uninjured control group without training.

3.4 Place each animal in the respective harness (**Figure 1**) and fasten harnesses to the body weight support mechanism above the treadmill *via* alligator clips which are fastened to weight support springs (**Figure 2 and Figure 3**). This requires the animal to be fixed in one spot on the treadmill, ensuring that they go in the designated forward direction and speed.

NOTE: Due to time and personnel constraints, the authors' lab conducts daily training in groups of twelve animals, three in each subset group.

3.5 Start the acclimation process following the previously published protocol[17]. Commence acclimation to LT (start of week 3 post-SCI) with a gradual treadmill exposure regimen, increasing from 10 min on Day 1 to the full target of 58 min over the first week (**Table 2**). Typically, by Day 4, the animals acclimate well to the training regimen. If an animal does not show progression by the third day of acclimation, the time would be reduced, and extra days added at a more gradual ramp-up (rare occurrence).

3.5.1 If an animal during the first day or two shows distress with confinement to the harness, remove it from the harness and treadmill and place back in its cage and give it two treats to help reinforce future compliance. The next day, place the animal in the harness and weight support system again for 10 min. On subsequent days, increase the duration by 20 min initially then continue to increase the training duration daily to achieve full training by Day 10.

3.6 Follow the detailed training regimen provided in **Table 2**.

3.6.1 Due to limited hind limb use post-injury, rats in the QT group will require manual facilitation for proper paw placement while stepping on the treadmill. Use one finger on each hand (commonly the third digit) to aid in hip/waist support. When the animal requires further assistance in stepping, use this same finger to apply pressure above the knee to initiate stepping. If necessary, use a separate finger (commonly the fifth digit) to aid the foot in stepping.

NOTE: The amount of body weight support needed varies from animal to animal and changes as training progresses. The spring support system gives enough assistance to keep the animal positioned for a proper gait. Further support is provided as needed by the trainer per above. Note that a key element of LT is functionally appropriate paw placement for stepping and interlimb coordination that is promoted by the trainer and is independent of the support system.

3.6.2 For the FT exercise group, adjust the body weight support system to slightly elevate the hind limbs to ensure no sensory stimuli to the paws and no weight bearing is occurring through contact with the treadmill.

NOTE: The FT group serves as an exercise and metabolic control, similar to that of a hand-crank exercise in human activity-based training studies.

3.6.3 Have the NT group harnessed and attached to the body weight support system in a similar fashion as the QT and place the NT group near the QT group on a stationary surface (**Figure 2 and Figure 3**).

NOTE: The NT group receives no activity and controls for any potential effects of being harnessed for an extended time period.

3.6.4 A home cage group can serve as an additional control. Transport these animals to the training facility as an additional step for this group.

3.7 By Day 7 -10 following the start of LT, train each animal once daily, every day until the day of termination of the study. Following each day of training, give each animal a sugary treat to reinforce compliance. Continue daily LT on animals following the 1 h regimen provided in **Table 2** for the duration of the study (*e.g.*, 8-12 weeks to mimic the approximate 80 one-hour sessions that are done in clinical studies)[9].

4. Euthanasia and Tissue Collection

4.1 Administer a lethal dose of anesthesia to the animal (*e.g.*, urethane or a ketamine/xylazine mixture at least twice the dosage, based upon weight, that would be used to achieve a surgical depth of anesthesia).

4.2 When the heart is just barely beating, immediately begin perfusing the animal in a dedicated fume hood first with cold heparinized saline followed by cold, 4% paraformaldehyde solution.

4.2.1. Begin by using surgical scissors to make an incision across the diaphragm, exposing the thoracic cavity. Continue to cut through the ribcage rostrally on both sides, removing the ribcage. Insert the perfusion needle into the left ventricle of the heart and clamp needle with hemostats, then clip the right atrium.

4.2.2. Using a perfusion pump mechanism, allow the cold heparinized saline to flow through the animal's blood vessels. Once clear saline flows from the right atrium, switch over to the cold 4% paraformaldehyde solution, until the body has stiffened.

4.3 Remove necessary tissue such as kidney, bladder, colon, brain, sensory ganglia, and spinal cord, and store in 4% paraformaldehyde for up to 48 h at 4 °C. After 24 - 48 h, move tissue to 30% sucrose and store at 4 °C.

4.4 Move collected tissue to a 30% sucrose/phosphate buffered cryoprotectant solution until tissue is ready for cutting. To cut tissue, embed in a tissue freezing compound and cut on a cryostat at desired thickness depending on the type of tissue used (*e.g.*, 35 µm for brain and spinal cord tissue, 5 - 7 µm for organ tissues).

REPRESENTATIVE RESULTS:

Following this training protocol, it has been documented that only the QT animals demonstrate superior locomotor function when compared to the other groups[18]. However, due to the nature of our lab, our primary focus is to investigate non-locomotor benefits of activity-based task-specific training (ABT), including bladder, bowel, and sexual function. For instance, we have previously published data that shows LT results in an exercise-induced reduction of polyuria in both QT and FT groups of SCI rats (**Figure 4**)[17]. Also, an injury-induced decrease in transforming growth factor-β (TGF-β) expression in the kidney's, indicative of an altered immune response, was not seen in QT and FT groups, which had TGF-β levels similar to sham (no injury) animals. In the same study[17], awake cystometry was performed before euthanasia and tissue collection. The maximum amplitude of bladder contractions during void cycles was not significantly different across sham, QT, and FT groups, while NT groups remained significantly altered. Together, these data indicate a positive exercise outcome on kidney health and bladder function, thus improving urinary function after SCI.

The mechanisms underlying polyuria within the SCI population is currently not clear but is likely multi-factorial[32]. Some have hypothesized, for example, that pooling of fluid in the lower limbs while SCI individuals are in a wheelchair can lead to fluid overload and increased fluid elimination during postural shifts (such as moving from sitting to lying)[33]. Such an explanation does not hold for the pre-clinical model, which has led us to focus initially on arginine vasopressin (AVP), the hormone which controls fluid homeostasis in the body and can be modulated with exercise. AVP controls fluid homeostasis through activation of the V2 receptor in the kidneys which facilitates water resorption from the renal collecting ducts[34]. Preliminary evidence from a pilot experiment (chronic time-point with one lesion severity – 210 kdyn impact force) indicate a beneficial effect of exercise (LT and FT) on V2 receptor levels in the rat kidney (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Custom-made harnesses sized for male Wistar rats. Both QT and NT animals are placed in the same type of jacket (**A**) allowing for the use of hind limbs in the case of QT animals. There are additional straps sewed onto the harness used for FT animals (**B**) to raise up the hind limbs, assuring no body weight support. The large hook-and-loop material portions of the harness allow for easy adjustments to different sized animals and to any changes in the size of an individual animal over time.

Figure 2: Training station setup. Body weight support mechanism surrounding the treadmill for either NT (far left), QT (middle), or FT (right) groups.

Figure 3: Training station with animals. Top (**A**) and (**B**) side views showing body weight support mechanism and location of attachment support clips to the harnesses. Note that the hind limbs of the FT animal (**B**) is raised and off the treadmill belt. Inset (**C**) portrays a closer view of the clip fastened to the harness.

Figure 4: ABT effects on rat polyuria after SCI. The total volume of urine output (**A**) increased after SCI (*; $p < 0.05$) and returned closer to baseline after 9 weeks of LT training in both QT and FT groups but remained increased in the NT group relative to the trained groups (#; $p < 0.05$). All groups demonstrated increased urine output compared to baseline at 9 weeks and increased void volume (**B**). It is important to note that the number of voids (**C**) and the amount of water intake (**D**) remained the same across all groups. Values are means ± standard error. This figure is republished with author permission[17].

Figure 5: ABT effects on rat kidney. Western blot results for rat kidney levels of V2 receptors in 5 groups of 4 rats each (20 total), showing expression levels for the protein bands provided in panel **A** and group mean densitometry analysis results of the bands (using ImageJ; OD = optical density) in panel **B**, indicating a significant (*; $p < 0.05$) decrease in receptors at a chronic time-point (12 weeks) post-SCI and no decrease relative to baseline (sham surgical controls) for groups receiving 10 weeks of one-hour daily ABT. Error bars represent standard error.

Table 1: Anesthesia dosage chart based upon individual animal's weight.

Table 2: Training regimen of speed settings the treadmill should be on corresponding to the time spent at each speed.

DISCUSSION:

Our methods of ABT on rats after SCI is a novel therapeutic intervention. While other methods of exercise and step training in animal models may exist[35-37], this method mimics LT carried out clinically in the SCI human population, where we have seen promising results[23]. With the combination of our setup, regimen, and use of control animals, the results obtained from utilizing our training paradigm will help to understand the benefits of ABT after SCI. Future applications

of this protocol include observing the described outcomes of ABT at different training timeframes as well as the effect that ABT has on recovery from different levels and extents of injury.

One limitation of this design is the length of time for such experiments. Given that our training regimen for each animal requires 1 hour per day, every day for 10 weeks, substantial personnel time and an organized schedule is a necessity. An important aspect that requires special attention involves the FT group, which has unique harnesses with hook-and-loop material straps to secure the hind limbs above the treadmill for the elimination of weight support. It is important to ensure that the animal does not receive weight support, which is why a platform is not positioned under the rat's hind paws. In addition, as previous studies have indicated that the sensory input is a principal driver of locomotor system plasticity in the spinal cord[38-40], there is a constant need of handling the QT group to assist with stepping much the same as physical therapists in the clinical setting.

An important modification made to the commercially available treadmill system used for the animals was reversing the polarity. After exposing the motor, the positive and negative wires were switched which reverses the direction the treadmill moves. This allows for more space and easier access to reach and help train the animals (the system comes with a shock grid at one end that is designed to prevent non-harnessed, spinally intact animals from stepping off the treadmill belt).

ACKNOWLEDGMENTS:

The authors acknowledge Drs. Patricia Ward, April Herrity and Susan Harkema for their input and guidance, Christine Yarberry for surgical assistance, Yangsheng Chen, Andrea Willhite and Johnny Morehouse for technical assistance and Darlene Burke for assistance with statistics and behavioral assessments. Funding support for this work was provided by the Department of Defense (W81XWH-11-1-0668 and W81XWH-15-1-0656) and the Kentucky Spinal Cord and Head Injury Research Trust (KSCHIRT 14-5).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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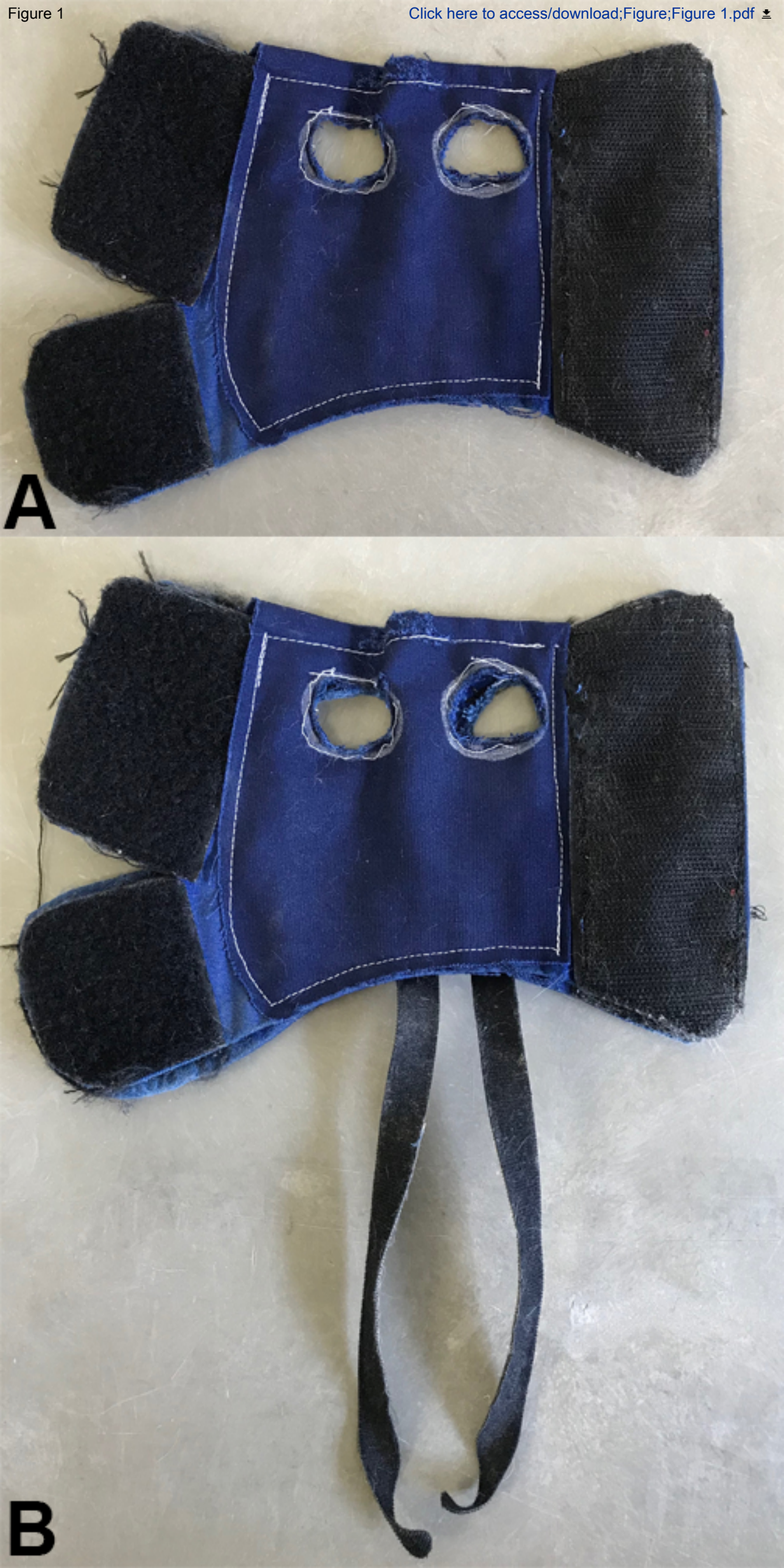
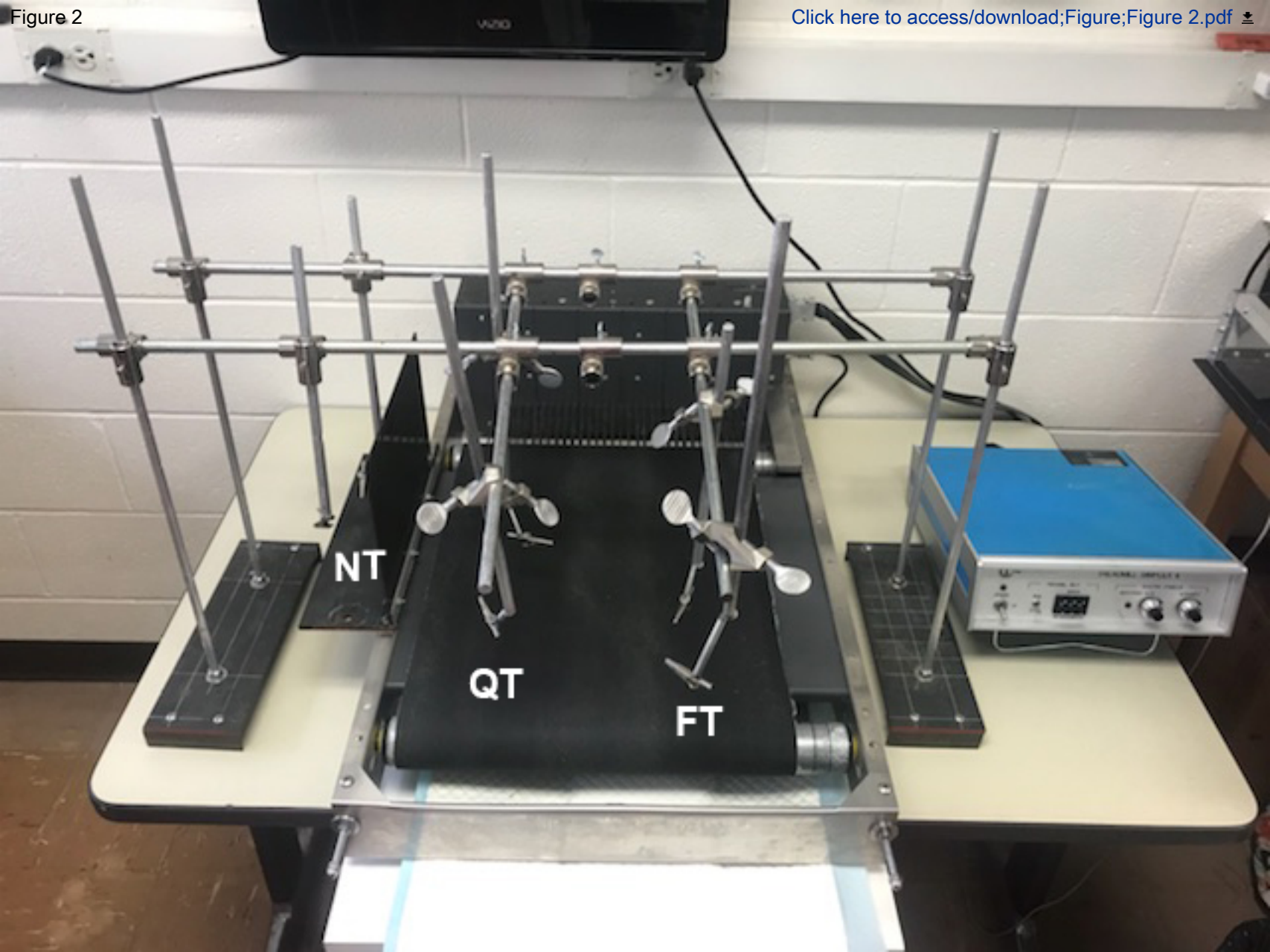


Figure 2



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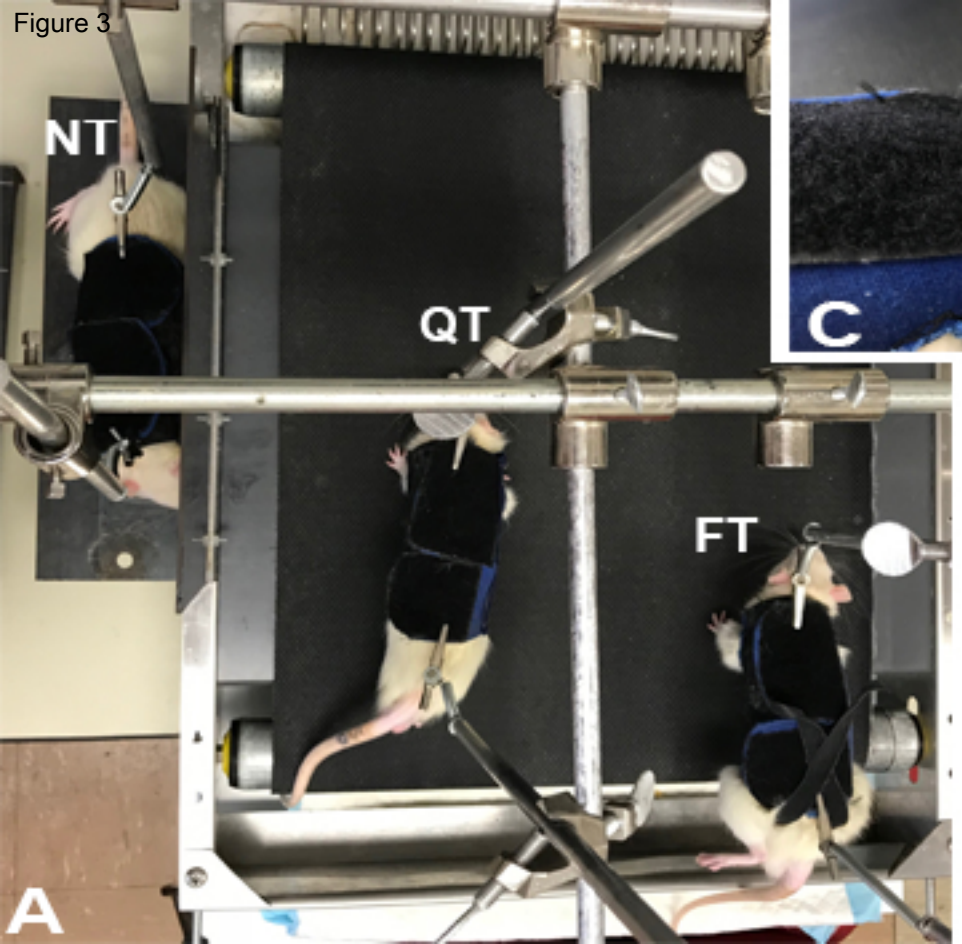
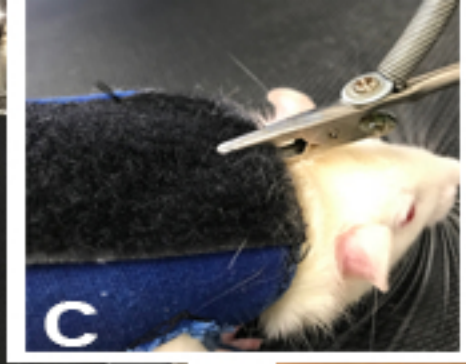




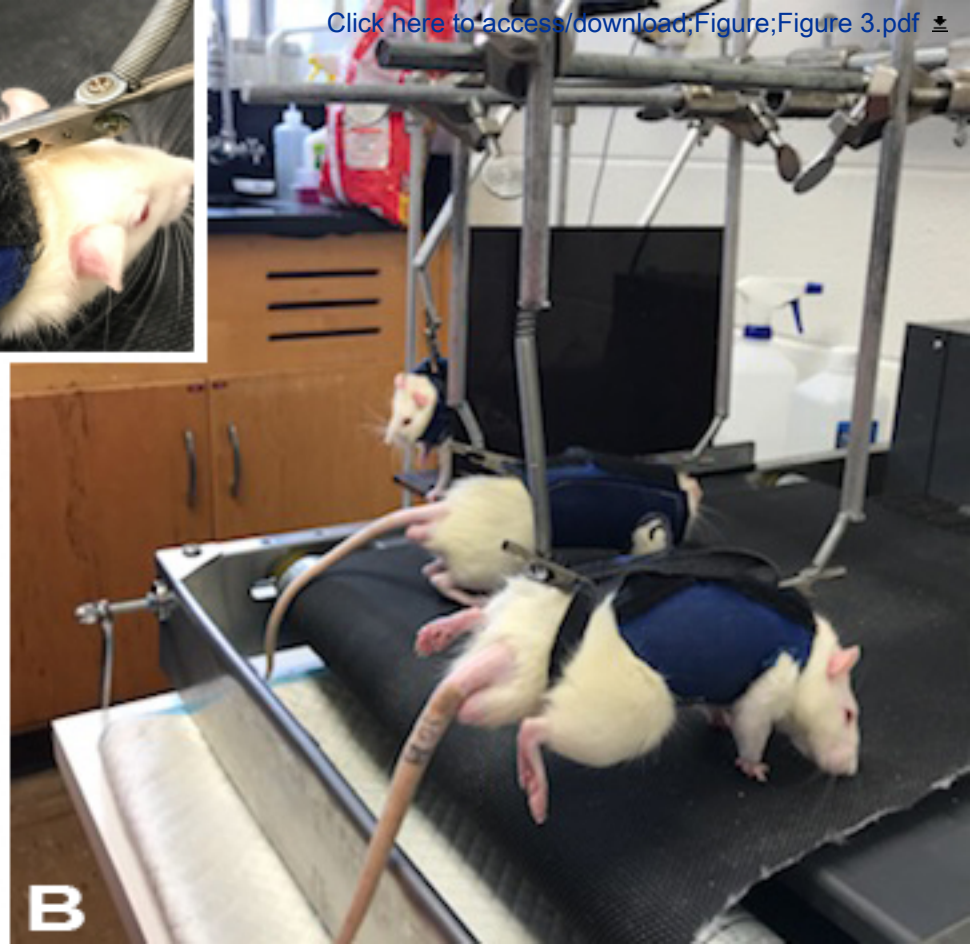


Figure 4

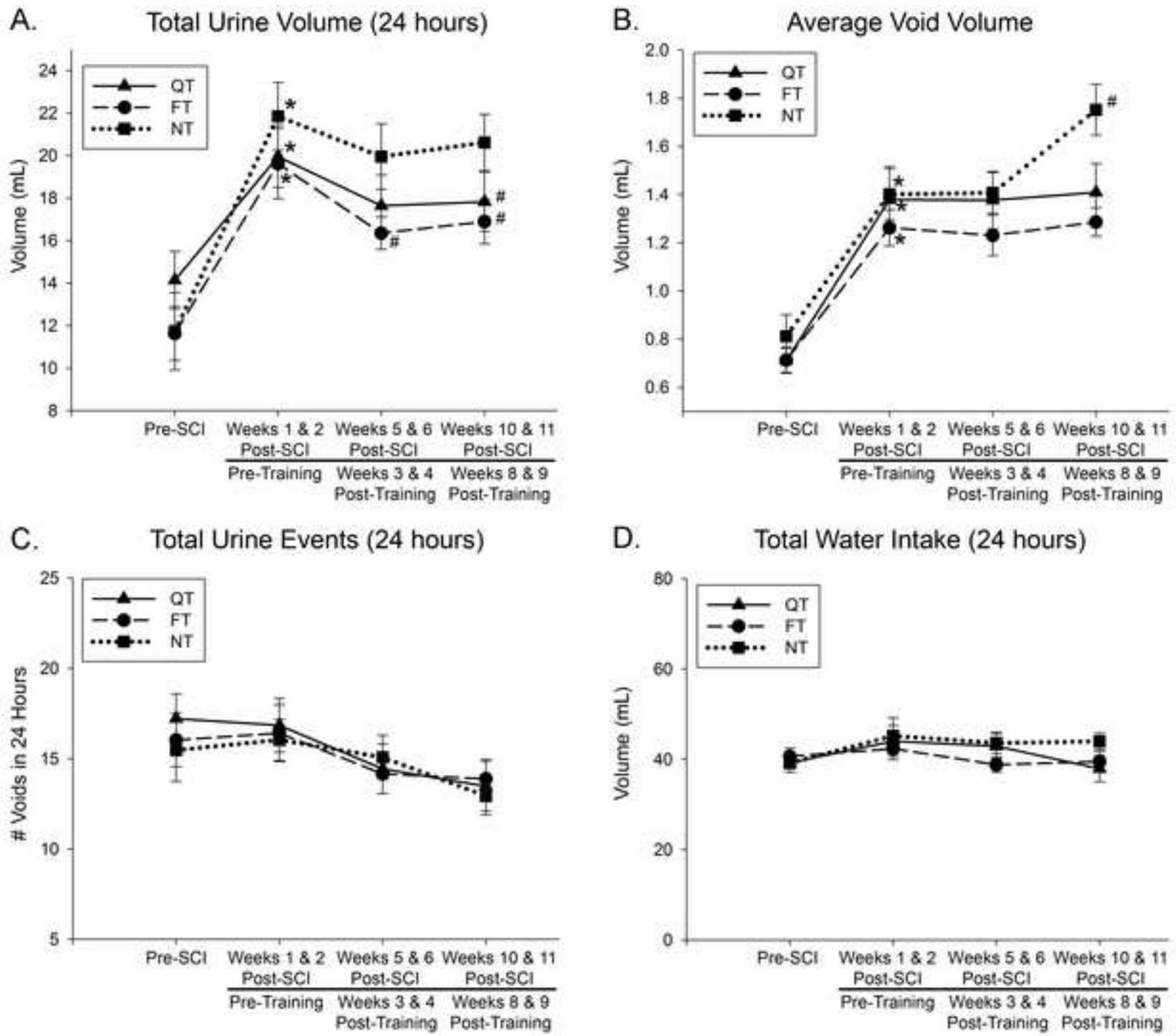
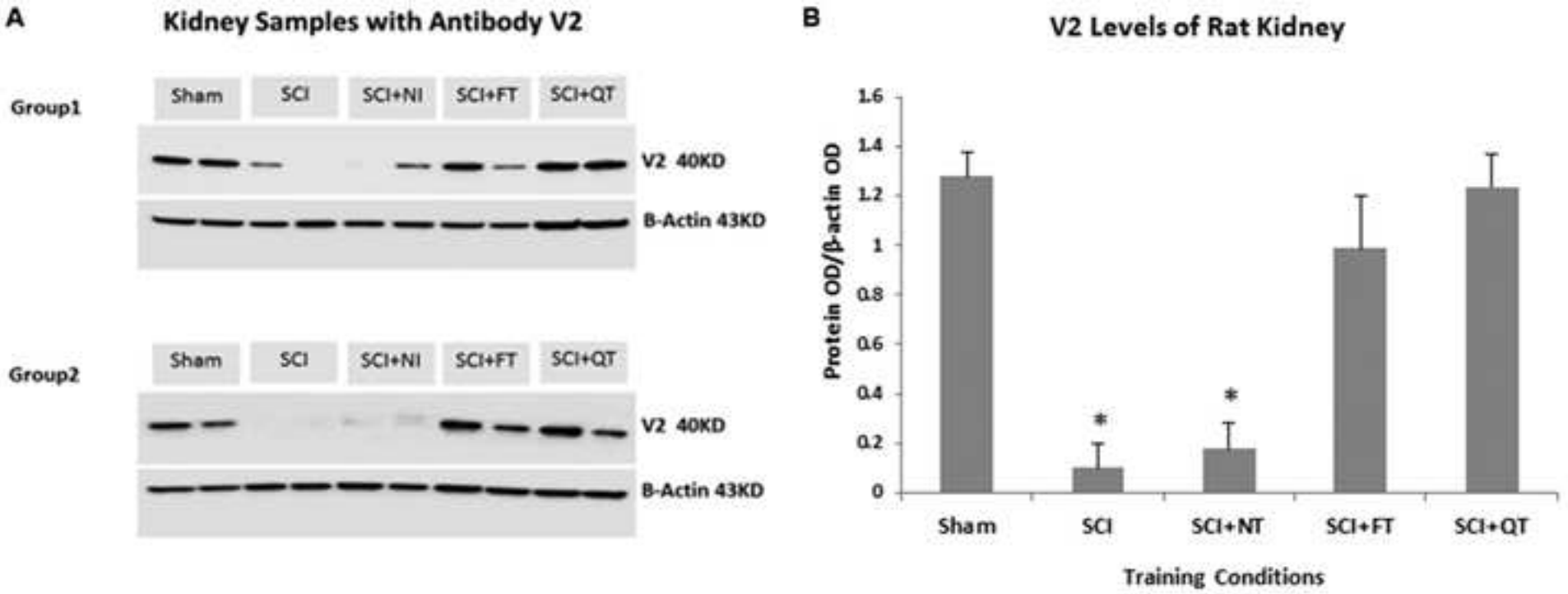


Figure 5



Ketamine/Xylazine Dose Chart

Effective dose: ***Using 100 mg/mL ketamine stock and 20 mg/mL xylazine stock***

80 mg/kg ketamine
10 mg/kg xylazine

1.0 mL mixture Injection = 0.62 mL ketamine stock (100 mg/mL) + 0.38 mL xylazine stock (20 mg/mL)

Animal Weight (g)	Mixture Injection (mL)	Animal Weight (g)	Mixture Injection (mL)
100	0.13	275	0.36
105	0.14	285	0.37
110	0.14	290	0.38
115	0.15	300	0.39
120	0.16	305	0.4
125	0.16	310	0.4
130	0.17	315	0.41
135	0.18	320	0.42
140	0.18	325	0.42
145	0.19	330	0.43
150	0.2	335	0.44
155	0.2	340	0.44
160	0.21	345	0.45
165	0.21	350	0.46
170	0.22	355	0.46
175	0.23	360	0.47
180	0.23	365	0.47
185	0.24	370	0.48
190	0.25	375	0.49
195	0.25	380	0.49
200	0.26	385	0.5
205	0.27	390	0.51
210	0.27	395	0.51
215	0.28	400	0.52
220	0.29	410	0.53
225	0.29	420	0.55
230	0.3	430	0.56
235	0.31	440	0.57
240	0.31	450	0.59
245	0.32	460	0.6
250	0.33	470	0.61
255	0.33	480	0.62
260	0.34	490	0.64
265	0.34	500	0.65
270	0.35	510	0.66

Table 2

Training Time (min)	Speed (cm/s)	Duration (min)
0-1	6	1
1-2	8.4	1
2-3	10.8	1
3-8	13.2	5
8-13	10.8	5
13-28	13.2	15
28-33	10.8	5
33-38	6	5
38-43	8.4	5
43-58	13.2	15

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Exer-3R treadmill	Columbus Instruments		reversed polarity of the motor
Body weight support system	N/A	N/A	modified spring scales with alligator clips
Rat harness	N/A	N/A	Our harnesses are custom made; please refer to Figure 1 for visual.
Infinite Horizon (IH) impactor device	Precision Systems and Instrumentation	Model 0400	
Ketamine HCl	Hospira	NDC 0409-2053-10	
Xylazine (AnaSed Injection)	Akorn Animal Health	NDC 59399-110-20	
Meloxicam (Eloxiject)	Henry Schein Animal Health	NDC 116695-6925-2	
Gentamicin Sulfate (GentaFuse)	Henry Schein Animal Health	NDC 11695-4146-1	
urethane, 97%	Argos Organics	CAS 51-79-6	
4-0 monofilament suture kit (4-0 Ethilon Nylon Suture)	Ethicon, LLC	205016	
Michel suture clips (9mm Auto Clips)	MikRon Precision, Inc.	1629	
Heating pad	Mastex Industries, Inc	Model 500	
Tootie Fruitys cereal	Malt O Meal		For training reward
Male Wistar rats	Envigo		
Size 10 surgical scalpel blades	Miltex	SKU: 4-110	

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Author(s):	Jason H Gumbel, Casey J Steadman, Robert F Hoey, James E Armstrong, Jason D Fell, Cui Bo Yang, Lynnette R Montgomery, Charles H Hubscher

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CORRESPONDING AUTHOR

Name:	Charles H Hubscher
Department:	Anatomical Sciences and Neurobiology; Kentucky Spinal Cord Injury Rehabilitation Center
Institution:	University of Louisville, Louisville, KY, USA
Title:	Professor and Vice Chair
Signature:	 Date: 8/22/16

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Dear JoVE Peer Review,

Thank you for your consideration of our manuscript JoVE58983 titled “Activity-Based Training with Spinal Cord Injured Wistar Rats.” We would also like to thank the reviewers and editors for their input and valuable time given toward improving the manuscript. We have read the comments and have revised the manuscript to address the reviewer’s concerns. In response to the reviewers’ suggestions, we have added more detail where necessary, fixed any grammatical errors, and expanded more description within the Representative Results and Discussion sections. Our revisions are discernable through track changes. Here we would like to respond individually to each of the editorial and reviewer’s comments:

Editorial Comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: The manuscript has been proofread for spelling and grammatical errors.

Editorial Comment: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Response: According to the American Journal of Physiology, Renal Physiology, and the American Physiology Society (APS), republication of figures, such as Figure 4 in our manuscript is allowed without charge or requesting permission so long as a full citation of the source is provided. In the figure legend we have noted that “This figure is republished with author permission.[17]” https://www.physiology.org/author-info.permissions

Editorial Comment: Keywords: Please provide at least 6 keywords or phrases.

Response: We have added “rehabilitation” and “exercise therapy” to our list of keywords.

Editorial Comment: Please expand the Summary to briefly describe the applications of this protocol.

Response: We have added a brief statement “Investigators are able to assess training effects on SCI rats using this protocol.” To describe our application of this protocol.

Editorial Comment: Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: We have added in more detail to the protocol steps where we thought was necessary, specifically including the following editorial comments:

Editorial Comment: 1.1: Please specify the age, gender and type of animals used. How many animals are used?

Response: We have revised step 1.1 (line 90) to say "Transport the rats (we use adult male Wistar rats - ~50 days of age initially; 200-225 grams) to a quiet room that is dedicated for training. Due to time and personnel constraints, we conduct our daily training in groups of twelve animals, three in each subset group (quadrupedal trained, forelimb only trained, non-trained, and home cage) that are randomly assigned after injury but prior to the start of training."

Editorial Comment: 1.3: For how many days and how many times per day are the animals trained? Please specify how to train the animal that does not acclimate well. For instance, start with what training time/speed/duration?

Response: We have revised step 1.3 to include more detail on how many days (7) a week and how many times a day (once) the animals are trained. Additional detail has also been added on what to do when an animal does not acclimate well.

Editorial Comment: 2.2: How is the general antibiotic given to the animal?

Response: Originally, we noted that the animals are given antibiotic "s.c." meaning subcutaneously. We have revised to write out "subcutaneously" rather than using shorthand.

Editorial Comment: 2.4: How large is the incision?

Response: We have added in to make an estimated 5 cm incision.

Editorial Comment: 2.5: Please describe how to perform laminectomy.

Response: We have added the following to step 2.5 (line 125): "For mid-thoracic contusions, expose the T8/T9 level of spinal cord via removal (with rongeurs) of the overlying T7 vertebral lamina."

Editorial Comment: 4.1: Please specify the anesthesia and dose used.

Response: Anesthesia for overdose is lab-dependent, but we stated the following based upon what we do: "Administer a lethal dose of anesthesia to the animal (at least twice the dosage, based upon weight, that would be used to achieve a surgical depth of anesthesia). Typical anesthesia drugs we use include urethane or a ketamine/ Xylazine mixture."

Editorial Comment: 4.2, 4.3: Please describe how this is done. What is the temperature for storing these tissues?

Response: We have described what we do in both 4.2 and 4.3, although there are many ways to go about saving tissue from perfused animals.

Editorial Comment: 4.4: Please specify how the tissues are later processed.

Response: We have revised this step to include how our tissue is prepared for histological analysis.

Editorial Comment: 6. In the protocol, please add steps that describe how to collect the data presented in Figures 4 and 5.

Response: Re Figure 4, the use of metabolic cages is not part of the training protocol; it is something our lab did to assess training effects on bladder function and is being used as an example that references the article containing the protocol. Re Figure 5, the collection of kidney tissue is described. The Western Blot protocol is standard and referenced (again, lab-specific and not part of the training video for the current JOVE article).

Editorial Comment: 7. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However, for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

Response: Figures 1, 2 and 3 are referenced in the Protocol as they deal with training of the animals, the topic of the video. Figures 4 and 5 are examples of how training and exercise can benefit function (in the case of spinal cord injury, we show benefits to the urinary system, although there are many other benefits – locomotion, cardiovascular function, etc…). The intent is the have this video show how training is done as different labs use it or will want to use it for different purposes (our benefits to the urinary system is just an example based on what we're studying; it's not meant for others to do as part of the protocol; they will apply training/exercise for their own purposes and whatever condition they are studying and will remove tissues relevant for their system of interest).

Editorial Comment: 8. Figure 4: Please change "ml" to "mL". Please label the different panels and describe them in the figure legend. Please define the * and # symbols as well as error bars in the figure legend. Please move the discussion of the figure to the Representative Results section. Please note that Figure 4 shows that after 9 weeks of LT training, total urine volume seems to be higher than baseline if baseline refers to the Pre-SCI value. Please check and clarify.

Response: Changes have been made to Figure 4 and the legend as requested. Yes, urine volume is significantly improved but remains significantly above pre-injury baseline. This is now pointed out in the legend and the discussion of it referenced in the paper.

Editorial Comment: 9. Figure 5: Please provide a figure with higher resolution. Please define error bars.

Response: Higher resolution figure now provided, and error bars now defined in legend.

Editorial Comment: 10. Discussion: Please describe any future applications of the technique.

Response: Lines 316-319 have been revised to describe our intended future application of this technique.

Editorial Comment: 11. Reference #12: Please spell out the journal name.

Response: The journal has been spelled out.

Editorial Comment: On line 90-91, the description of the pre-contusion acclimation training makes it sound like it very easy to do and naïve animals are quick to learn it. If that is the case, fine, but our experience has shown that animals should be eased into the harness in the beginning, then later, we introduce them while in the harness to the weight support mechanism. Only then do we turn on the treadmill and have them walk. If there are similar experiences, perhaps that authors can provide more detail so as not to give the impression of it being fairly easy to do.

Response: Edits have been made to reflect the different procedures used during acclimation, including clarifying that this is done post and not pre-injury.

Editorial Comment: Around line 146, the training details should include whether the set up allows any forward/backward movement on the treadmill belt. It seems the animals are fixed in one spot? If so, please address this as it has some implications for training.

Response: A few edits have been made for clarity but as this is a video journal, all details will be evident in the accompanying video.

Editorial Comment: Around line 157, "more severely injured rats" implies (rightly so) that there may be some variability in recovery after contusion. Do the authors have some criteria for removing animals from the study, i.e. those who may not appear injured or appear too injured?

Response: Wording fixed. All rats in the separate groups receive the same contusion impact forces; random assignment just prior to training accounts for potential variability in spontaneous recovery. Our injury inclusion/exclusion criteria are study-specific and is not relevant to the training video.

Editorial Comment: I assumed there would be a video given this is JOVE, if there is one available, it may be helpful to include the training of rats, especially how the manual assistance is provided.

Response: The written manuscript protocol is submitted prior to videotaping (video on how the training is done).

Editorial Comment: 1.3 - The final sentence "reduce the time and increase training by only 10 min/day" needs to be clarified. It is not immediately clear how and by how much.

Response: We have revised the steps to include more detail on how to train animals that are not initially compliant.

Editorial Comment: 2.4 - There is a typo at the end of the first line "and ith a #10"

Response: The typo has been corrected to say “with a #10…”

Editorial Comment: 3.6.1 - This explanation should include a corresponding figure for clarification

Response: It is our belief that the main purpose of submitting to JoVE is to demonstrate this aspect of our training protocol via a video demonstration. Therefore, a figure would be redundant and not as helpful.

Editorial Comment: Include a discussion on how the body weight support system cannot be controlled precisely by this system and how this could affect locomotor performance.

Response: The following edits have been added to address this point: The amount of body weight support needed varies from animal to animal and changes as training progresses. The spring support system gives enough assistance to keep the animal positioned for a proper gait. Further support is provided as needed by the trainer. A key element of LT is functionally appropriate paw placement for stepping and interlimb coordination that is promoted by the trainer and is independent of the support system.

Editorial Comment: In the discussion of the influence of afferent information on functional recovery, how skin afferents activated by the straps holding the hip could affect spinal circuits?

Response: Edits made to emphasize the non-trained control group that wears a training jacket.