

Research paper

A standardized in vivo platform for preclinical screening of therapeutic interventions in rat thoracic spinal cord injury

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ABSTRACT

Reproducibility remains a challenge in spinal cord injury (SCI) research, and the absence of standardized pre-clinical models and outcomes slows clinical translation of promising findings. Contract research organization CRO-based screening platforms partially address this in epilepsy and pain, yet no equivalent resource exists for SCI. Thus, the Christopher & Dana Reeve Foundation partnered with PsychoGenics, Inc. and an expert panel to develop a standardized, blinded screening platform using a rat mid-thoracic (T8) contusion model tested across four severities on Infinite Horizons impactor device (170, 200, 250, and 250 Kdyn+3 s dwell time; $n = 15$ /group). Outcome measures included open field locomotion assessed by Basso Beattie Bresnahan locomotor rating scale and NeuroCube, an automated gait analysis system using computer vision and machine learning to quantify gait, as well as horizontal ladder, mechanical, heat and cold allodynia, and immunohistochemical assessment of the SCI. Most measures distinguished injury severities from Sham and several demonstrated graded effects across severity levels. BBB scores at 5 weeks mapped onto distinct locomotor milestones, while NeuroCube analysis captured compensatory adaptations, namely forward weight shift, wider base of support, and increased forelimb reliance. Hindlimb error rates on the horizontal ladder ranged from 22% to 95% and corresponded to SCI severity. Mechanical and cold allodynia occurred in all SCI groups, increasing with severity. Histologically, the spared tissue area and volume measurements are sensitive measures for distinguishing injury severity, correlating strongly with BBB, ladder, cold allodynia, and NeuroCube outcomes. This standardized platform reliably detects functional and histological differences across clinically relevant thoracic SCI severities. It offers a reproducible, objective resource for industry and academic investigators seeking to evaluate therapeutic candidates in the SCI discovery pipeline.

1. Introduction

As in many other areas of CNS research, the field of spinal cord injury (SCI) continues to struggle with the development of effective and reliable treatments. The translation of promising findings into meaningful functional recovery in persons with SCI reflects multiple factors, including the complex nature of SCI itself that affects motor, sensory and

autonomic functions as well as research-related factors like reporting bias and limited reproducibility and replicability. These issues are amplified by the entrepreneurial or academic non-SCI investigator's lack of accessibility to standardized, sensitive, pre-clinical models and outcome measures (Steward et al., 2012).

Over the past 2 decades, several initiatives led by federal and non-profit organizations have attempted to close this gap by funding

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programs to train new investigators in standardized SCI models, to support replication studies, and to codify commonly used data definitions across clinical and experimental SCI labs. The National Institute of Neurological Disorders and Stroke (NINDS) FORE-SCI program funded a series of replication studies, revealing that full replication was rarely achieved (Steward et al., 2012). More recently, NINDS established common data elements (CDEs) to harmonize data definitions across clinical studies, with pre-clinical CDEs currently under development (Biering-Sorensen et al., 2015). These efforts improved transparency in the dissemination of findings by highlighting the importance of seemingly small experimental details, but they did not increase the number of therapeutics in the pipeline.

The wide variety of published experimental models may act as a deterrent for non-SCI academicians, industry investigators or investors wanting to enter the SCI space, as the cost and time for standardizing and implementing SCI models and appropriate behavioral assays are often prohibitive (Cheriyian et al., 2014; Fouad et al., 2020; Scheff et al., 2003). Pre-clinical contract research organizations (CROs) can address some of the remaining barriers by offering specialized services including expertise and the infrastructure to perform in vivo experiments. The Epilepsy Therapy Screening Program (ETSP) and the Preclinical Screening Platform for Pain (PSPPP), two NINDS initiatives, and the Tuberous Sclerosis TSC Alliance have partially addressed similar challenges by establishing standardized preclinical screening platforms at CROs to accelerate therapeutic discovery in epilepsy, pain, and tuberous sclerosis complex, respectively (Iyengar et al., 2020; Kehne et al., 2017) (NINDS, 2026a; NINDS, 2026b). These programs provide a strong precedent and proof of concept that this model of pipeline acceleration can be successful. Thus, the Christopher & Dana Reeve Foundation with input from SCI experts, partnered with PsychoGenics, Inc. (PGI), a CRO experienced in high volume preclinical testing in neuroscience to establish a comparable in vivo platform tailored specifically for SCI therapeutics.

We adopted established rat mid-thoracic (T8) contusion SCI models ranging from mild to severe, combined with a comprehensive battery of behavioral and anatomical assessments to standardize an in vivo platform, to facilitate the evaluation of the therapeutic potential of interventions in the SCI discovery pipeline. The mid-thoracic contusion model was selected as the initial focus given its widespread use and extensive characterization in preclinical SCI research. Designed for the screening of large sample size rodent cohorts, this platform is transparent and provides consistent, and objectively generated data through blinded studies. In contrast to NINDS pre-clinical screening platforms, any therapeutic (small molecule, biologic, device) determined by the investigators to be ready for behavioral testing, can be evaluated here. Importantly, this platform was developed to be accessible and available to investigators with or without SCI expertise in academia or industry. We propose that this platform has the potential to streamline the SCI discovery pipeline and expedites selection of promising therapeutics for clinical trials.

2. Materials and methods

2.1. Animal subjects, experimental design, and scientific rigor

PGI is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All work was performed under PHS OLAW Assurance. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of PGI and carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Female, Sprague-Dawley rats, (12–15 weeks of age and 200–250 g, at the time of mid-thoracic injury) obtained from Inotiv (West Lafayette, Indiana) were housed in ventilated rat cages (395 × 346 × 213 mm) with a 12-h light/dark cycle. Food and water were provided ad libitum. Room temperature was maintained between 20 °C and 23 °C, with

relative humidity kept around 50%. Rats were assigned unique identification numbers (PGI ID and tail markings), and were examined, handled, and weighed prior to the study. Following SCI surgery, rats were housed in standard, static (non-ventilated), rat cages (480 × 267 × 210 mm), with Alpha-Dri bedding (Shepherd Specialty Papers, Milford, New Jersey), changed three times per week. Water bottles with extended tips were provided to ensure easy access to water. Before and after injury, rats were housed in pairs.

Sample size ($n = 15/\text{group}$) was determined by power analysis of pilot data (PGI, unpublished) using compounds previously reported as efficacious in similar mid-thoracic SCI models (Riluzole (Mu et al., 2000; Schwartz and Fehlings, 2001), Rolipram (Costa et al., 2013; Pearce et al., 2004)), with change in BBB score (Basso et al., 1995) as the primary endpoint. This analysis indicated that 15 rats per group were required to achieve adequate statistical power for drug discovery studies. After baseline behavioral assessments, rats that meet inclusion criteria (baseline BBB score = 21, average baseline paw withdrawal threshold (PWT) <14 g) were allocated to either Sham (laminectomy only) or one of four distinct T8 contusion severities (170 Kdyn, 200 Kdyn, 250 Kdyn, 250 Kdyn +3 s dwell) with behavioral deficits ranging from mild to severe. The mildest injury severity was 170 Kdyn rather than the more commonly used 150 Kdyn because 150 Kdyn thoracic contusions typically recover to the upper range of the BBB locomotor scale by approximately 4 weeks post-injury, where the scale is less sensitive to subtle locomotor differences and more susceptible to interobserver variability (Cao et al., 2005; Ferguson et al., 2004). Thus, 170 Kdyn was selected to preserve a mild injury phenotype while maintaining clear separation from sham controls and locomotor performance within a more discriminative range of the BBB scale. The most severe contusion was produced by prolonging the dwell time of the applied force rather than by increasing its magnitude, as forces exceeding 250 Kdyn (e.g., 300 Kdyn) are less reliably transmitted to the spinal cord. Experimental groups were assigned and balanced by pre-surgery body weight.

Locomotor and sensory behavior were assessed for 5 weeks post-injury by investigators blinded to experimental group as much as possible. (rats with SCI do look physically different than Sham groups, especially in the early time period post surgery). After behavioral assessments, spinal cords were harvested for immunohistochemical analysis. Plasma samples (baseline, Day 1, Week 1, and endpoint) and cerebrospinal fluid (CSF) samples (endpoint) were collected for later evaluation of biomarkers. The study design were performed as a partnership between the Christopher & Dana Reeve Foundation (with input from its SCI Model Expert Committee – authors MRD, KF and WT) and PGI. All experiments were conducted by PGI staff with significant input from members of the SCI Model Expert Committee (MRD, KF, and WT) in the form bi-weekly consultation meetings. PGI personnel had prior experience conducting thoracic SCI studies, including contusion injuries using the IH impactor, BBB assessments, horizontal ladder testing, von Frey and acetone assays, as well as other rodent models of neurological disease requiring microsurgical, behavioral, and immunohistochemical expertise. All personnel completed PGI's internal training and qualification program prior to participating in study activities. This program includes SOPs developed and approved by investigators with expertise in SCI research, procedure-specific instruction, supervised hands-on training, and competency-based qualification assessments. Personnel were required to demonstrate proficiency and reproducibility in each procedure before independently performing study-related activities.

A timeline of the experiment is summarized in Fig. 1 and Table 1. A total of 92 animals were enrolled as a single experimental cohort using a staggered enrollment schedule across 3 consecutive weeks where mid-thoracic SCI surgeries were performed. The distribution of rats into experimental groups on each surgery day is provided in Table 2. This study design enabled completion of all in vivo procedures within an 11-week timeframe. Immunohistochemical processing and analysis was conducted in the subsequent 8 weeks.

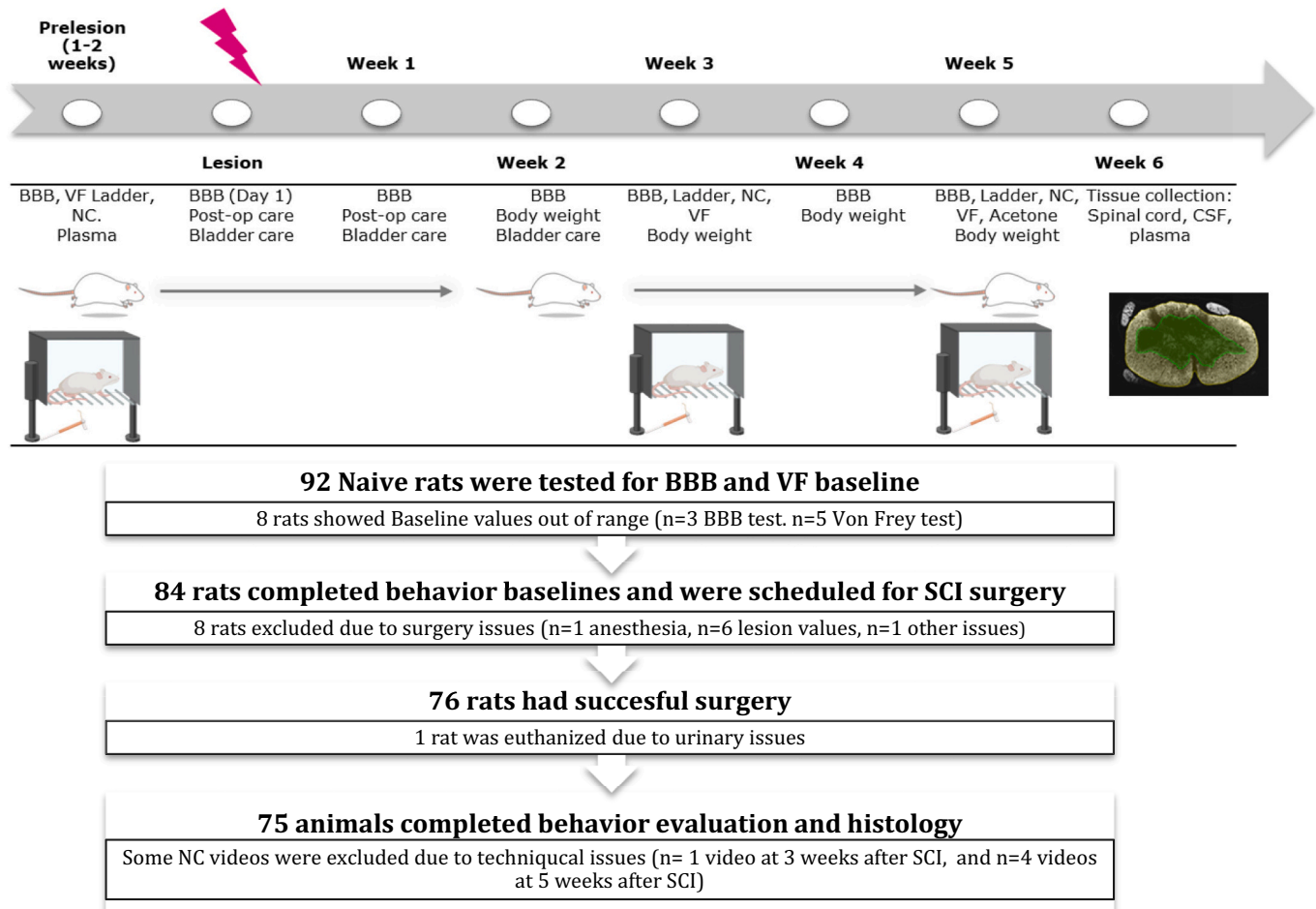


Fig. 1. Experimental timeline and study enrollment flow chart. Baseline behavioral assessments and plasma samples were collected prior to injury (Prelesion). BBB scores were evaluated on day 1 post-injury and weekly thereafter. The horizontal ladder, NeuroCube, and von Frey tests were conducted at Week 3 and 5 post-SCI, while the acetone test was performed at Week 5 only. The flow diagram summarizes animal enrollment and exclusions across experimental stages.

Table 1

Staggered experimental schedule across the 11-week study period.

Study week	Cohort 1	Cohort 2	Cohort 3
1	Animal arrival	–	–
2	Baseline testing	Animal arrival	–
3	SCI (Surgery Days 1–3); corresponding Day 1 BBB assessments	Baseline testing	Animal arrival
4	Post-SCI Week 1	SCI (Surgery Days 4–6); corresponding Day 1 BBB assessments	Baseline testing
5	Post-SCI Week 2	Post-SCI Week 1	SCI (Surgery Day 7); Day 1 BBB assessment
6	Post-SCI Week 3	Post-SCI Week 2	Post-SCI Week 1
7	Post-SCI Week 4	Post-SCI Week 3	Post-SCI Week 2
8	Post-SCI Week 5	Post-SCI Week 4	Post-SCI Week 3
9	Tissue collection	Post-SCI Week 5	Post-SCI Week 4
10	–	Tissue collection	Post-SCI Week 5
11	–	–	Tissue collection

2.2. Spinal cord injury model

Contusive SCI at the T8 level was produced using the Infinite Horizon (IH) Impactor (Precisions Systems and Instrumentation, LLC, Lexington, Kentucky) as previously described (Scheff et al., 2003). The surgery was

performed with aseptic procedures in a designated area under deep anesthesia. Anesthesia was induced in a chamber using 1.5 l/min of oxygen and 4–5% isoflurane for induction, 2% for maintenance. To maintain body temperature, rats were placed upon a homeothermic blanket system with continuous feedback from a rectal thermometer (RightTemp, Kent Scientific, Connecticut, USA). First, a laminectomy was performed at the thoracic (T8) vertebral level. The spinal column was stabilized by securing the lateral processes of T7 and T9 vertebral bodies using micro-Adson forceps (0.8 mm tips), which were part of the IH impactor assembly. The forceps were mounted onto custom-made micromanipulators allowing for movement along three axes, enabling precise positioning of the exposed spinal cord in a level and stable plane. The standard, cylindrical shape, 2.5 mm diameter impactor tip was centered in the laminectomy site and raised 5 mm from the surface of the cord. The IH device force and dwell time parameters were set according to group allocation (170, 200, 250 Kdyn or 250 Kdyn +3 s dwell). The 3 s dwell time adds a short compression by increasing the time the probe is in contact with the spinal cord, producing a more severe injury while remaining within the reliable operating range of the IH device. This added dwell time may better represent human SCI which often has both contusive and compression injury. (Mattucci et al., 2019) To ensure the consistency and quality of the lesions the force and displacement impact graphs produced by the IH device were reviewed and rats excluded if any unusual morphology (i.e., double peak or plateau) was observed in the force graph, or when the displacement was read as “0” (impactor-tip/tissue interface not properly detected). Following injury, muscles

Table 2

Distribution of animals across SCI surgery days during staggered enrollment.

Enrollment week	Surgery day	Sham	170 Kdyn	200 Kdyn	250 Kdyn	250 Kdyn + 3 s	Excluded during surgery	Total surgeries
Week 1	Day 1	2	2	4	1	1	3	13
	Day 2	1	1	1	3	2	2	10
	Day 3	2	2	1	1	3	1	10
	Day 4	3	3	3	3	2	0	14
Week 2	Day 5	3	3	2	3	3	0	14
	Day 6	2	2	2	2	4	1	13
	Day 7	2	2	2	2	1	1	10
Total		15	15	15	15	16	8	84

were closed in layers using absorbable 4–0 Ethicon vicryl suture, and skin closed with wound clips which were removed 7–10 days after SCI. The rats received post-operative treatment and care that included the administration of analgesics for 2 days (buprenorphine 0.03 mg/kg, subcutaneous, SC, twice per day), fluids for at least 3 days (Ringer electrolyte solution, 6–8 ml SC, twice per day), and prophylactic amoxicillin diet (Mod LabDiet® 5058 w/0.12% amoxicillin) for 7–10 days after SCI. Sham-operated rats received the same pre and post operatory procedures, a laminectomy was performed at the T8 level, but no spinal cord lesion was produced. After surgery, rats were housed on standard non-ventilated cages. For 7–10 days post-surgery, half of each cage was positioned over a temperature-controlled heating pads (37–38 °C), allowing rats to self-regulate their environment. Water and food were easily accessible. Urinary bladders were manually expressed twice a day, by gentle abdominopelvic compression, until rats recovered spontaneous voiding. While exclusion of male rats in this study minimizes rates of attrition due to complications from urinary tract infections after SCI, proper validation of therapeutic efficacy in preclinical models requires incorporation of both males and females. This limitation is of particular importance for preclinical models of SCI such as the one highlighted in this study, as epidemiological data has indicated that human males experience traumatic SCI at over three times a higher rate than females (Lu et al., 2024). Furthermore, sexual dimorphisms exist in SCI pathology and functional recovery in both rodent models (Guo et al., 2025; Walker et al., 2019) and within the clinical population (Benedict et al., 2026; Kirshblum et al., 2021), suggesting an intervention demonstrating therapeutic efficacy in females may yield different results in males, and vice versa.

The general health of the rats was monitored during bladder expression and behavioral testing. Health issues were discussed with a veterinarian if needed.

2.3. Outcome measures

2.3.1. Gross motor assessment using BBB open-field locomotor rating scale

Hind-limb (HL) locomotor performance was assessed using the open-field Basso, Beattie, Bresnahan (BBB) locomotor rating scale as described previously (Basso et al., 1995; Basso et al., 1996b). Each rat was placed in an open field arena (open enclosure, 4 ft. diameter,) and allowed to freely explore for 4 min. HL movements were assessed by two trained raters (blinded to group allocation) before injury (baseline), at Day 1 after T8 SCI, and then weekly for 5 weeks after SCI. BBB sub-scores were calculated from the full BBB assessment. Rats that did not achieve weight support (BBB score < 10) were considered to have a sub-score of 0 (Popovich et al., 1999).

2.3.2. Unbiased gait assessment using the neurocube platform

The NeuroCube® (NC) platform, is a PGI proprietary gait analysis system. It is an automated in-vivo high-throughput platform used to assess locomotor performance and gait in rodents utilizing computer vision and machine learning algorithms to automatically detect and quantify locomotion features (Alexandrov et al., 2015a, 2015b; Huang et al., 2022). Spontaneous locomotion was evaluated by allowing rats to freely explore the enclosed, circular, arena (30 in. wide) for 5 min

while their movements were recorded via undermount camera through a transparent floor. Paw images and processed videos were reviewed for quality control.

Digital videos were automatically processed and analyzed using the NC algorithm, generating a comprehensive database of raw output parameters. These included measures of animal activity (walking distance, walking time, and average walking speed), gait geometry and dynamics, paw morphometrics, body morphometrics, patterns and coordination. During each 5-min session, the NC system detected an average of approximately 80 steps per animal. Outcomes were automatically computed for each paw and further stratified by speed range (low: <30 cm/s; mid: 30–50 cm/s; high: >50 cm/s). Table 2 summarizes the parameters generated by the NC platform; no manual scoring, or calculations were performed. Given the bilateral nature of the T8 thoracic SCI model, left and right limb measurements were averaged to generate a single forelimb (FL) value and a single HL value for each rat at each timepoint.

2.3.3. Sensorimotor assessment using horizontal ladder test

Locomotor coordination and fine paw dexterity were assessed using the Horizontal Ladder test using established methods (Metz and Whishaw, 2002). The Horizontal Ladder test evaluates integration of sensory and motor function by assessing a rat's ability to accurately place their paws on irregularly spaced rungs. Rats were placed in an acrylic chamber (1 m length by 7 cm width) containing a runway with metal rungs spaced at 3 rungs per inch. To create a runway containing irregular spaced gaps, either one to three rungs were removed in a randomized pattern. The rung configuration was changed for each timepoint to minimize learning effects. A total of 3 crossings of a horizontal ladder per rat were acquired in a video (60 fps). Rats were not previously trained in the test. Videos were analyzed by counting footfall errors (instances where a paw failed to grasp a rung and slipped between bars) and the total number of steps taken by each paw (including step after a slip). Mean error rates were calculated separately for FL and HLs and expressed as a percentage of total steps. Rats unable to plantar step (BBB score < 10) were nevertheless able to traverse the apparatus using forelimb-driven locomotion. In this case, a HL error rate of 100% was assigned to reflect the inability to execute successful weight-bearing HL placements and to ensure inclusion of all animals in the analysis. Testing was performed before injury and repeated at Week 3 and Week 5 post-injury.

2.3.4. Mechanical allodynia assessment

Hind paw tactile sensitivity was evaluated using the methodology used for the NIH-sponsored Preclinical Screening Platform for Pain (PSP, <https://pspp.ninds.nih.gov/TestDescription/TestPWT>) (Chaplan et al., 1994). Rats were placed in an acrylic chamber (20.3w x 20.3d x 25.4 h cm) with a mesh floor and acclimated for 20 min prior to testing. A maximum of 9 filament applications was used to determine the 50% withdrawal threshold. Rats that do not respond to any filament (OOOOO), were assigned a threshold of 15.0 g, while rats that respond to all filaments (XXXXX) were assigned a threshold of 0.25 g. Paw withdrawal threshold was measured for both the left and right hind paws. Rats were tested before injury and at Week 3 and Week 5 after T8 SCI.

Rats that showed a baseline paw withdrawal threshold (PWT) lower than 14 g (average of left and right hind paw values) were excluded from the study to minimize variability associated with pre-existing tactile hypersensitivity.

2.3.5. Thermal sensitivity assessment

Thermal sensitivity to noxious heat was assessed using the Hargreaves test (Hargreaves et al., 1988). Rats were acclimated in acrylic chambers on a glass floor, and a focused radiant heat stimulus was applied to the plantar surface of each hind paw. Both hind paws were tested. Rats were evaluated prior to injury and at Week 2 and Week 4 following T8 SCI.

Cold allodynia was evaluated using the acetone evaporation assay by measuring aversive behaviors (paw lift or flinch responses) triggered by the evaporative cooling of a drop of acetone placed on the plantar surface of the hind paws (Yoon et al., 1994), following the methodology used for the NIH-sponsored PSPP program (<https://pspp.ninds.nih.gov/TestDescription/TestHPCA>). Rats were placed in white rectangular observation chamber made of acrylic (20.3w x 20.3d x 25.4 h cm) with black acrylic lids on an elevated, perforated platform and allowed to acclimate for a minimum of 20 min. Prior to the acetone application. Rats were tested by forming an acetone drop (~50 μ L) at the end of a 1 mL syringe and gently applying it to the plantar surface of the hind paw (left or right hindpaw). The hindpaw was then observed for approximately 20 s following application and either paw withdrawal, flinching or no response was recorded. The procedure was then repeated for the other hindpaw. A total of 5 acetone applications to each hindpaw were conducted with an interval of approximately 5 min. Between applications to the same hindpaw. A score for each paw was obtained. Rats were tested at Week 5 post T8 SCI.

2.3.6. Blood and CSF collection procedures

Tail vein blood samples (~200 μ L) were collected into commercially available K2EDTA tubes (Kitchen et al., 2021) before injury and at Day 1 and Week 1 after SCI. Within 15 min of blood collection, tubes were centrifuged for 10 min at 10,000–12,000 rotations per minute (RPM) at 4 °C. The supernatant (plasma) was extracted, placed on dry ice and stored at –80 °C until analysis.

At Week 6 post-SCI, rats were deeply anesthetized with isoflurane (4–5% isoflurane for induction, 2–4% for maintenance in 1.5 l/min of oxygen) and monitored until complete loss of reflexes in which all the responses to external stimuli cease. While under deep anesthesia, CSF was collected by cisternae magna puncture using a sterile 0.3 ml Insulin Syringes 31G x 6 mm needle. CSF samples were centrifuged for 1 min at 5000 RPM, frozen and kept at –80 °C until analysis.

The thoracic cavity was then opened to expose the heart, and blood was collected in K2EDTA tubes by cardiac puncture. Euthanasia was achieved by exsanguination during cardiac blood collection, followed by insertion of a blunt-end perfusion needle (oral gavage needle) into the left ventricle for transcardial perfusion. Transcardial perfusion was performed with 0.1 M PBS (200 mls) to flush remaining blood followed by 4% paraformaldehyde in phosphate buffer (PFA ~200 mls until muscles are stiff). After that, the spinal cord and brain were harvested and post-fixed for 48 h in 4% PFA at 4 °C. Blood samples were centrifuged for 10 min at 10,000–12,000 RPM. The supernatant plasma was collected, separated into two aliquots and frozen at –80 °C until use.

2.3.7. Immunohistochemistry procedures

After post-fixation, spinal cords and brains were transferred to 15% sucrose/PBS at 4 °C until the tissue sunk. Spinal cords were cut transversally into three blocks (the middle one contained the T8 SCI impact site), and those were embedded side by side in cryo-protective freezing medium (Tissue-Tek® O.C.T., Sakura®, 25608–930). Spinal cord segments were embedded and trimmed until a complete transverse section was obtained. Leica CM1950 cryotome at 10 μ m-thick and mounted to microscope slides. Sampling was then performed using a uniform and

systemic protocol where three consecutive sections were collected, followed by the discarding of the subsequent 22 sections, resulting in a sampling interval of approximately 250 μ m between analyzed planes. The collection was extended throughout the entire sample for 40 planes. Sections were stored at –20 °C until used for staining. Systematic random sets of sections, one slide per sampling plane containing three sections per sample (63 sections), were labeled for Fluoromyelin (FluoroMyelin™ Red Fluorescent Myelin Stain, Invitrogen; F34652). Sections were incubated for 30 min at room temperature, washed 5 $\times$ using PBS and then counter-stained with DAPI (Millipore, 10236276001), washed and coverslipped. Whole-slide imaging was conducted on a Zeiss AxioScan Z1 slide scanner microscope equipped with LED illumination; corresponding narrow band filter sets fitting to each dye, and a sensitive Orca Flash 4.0 B&W camera at 10 $\times$ magnification (mounted with 1 $\times$ opto-coupler thus no demagnification). Delineations and quantifications were performed on Image Pro Premier (IPP v9.1.5 or higher) from Media Cybernetics. To estimate lesion volume within approximately 3 cm of spinal cord, eleven consecutive sampling planes encompassing the lesion epicenter were spaced 250 μ m apart, while the remaining planes were spaced 750 μ m apart, covering a total length of 2.8 cm. For each cross-section, total cross-sectional area and lesion area were manually delineated as regions of interest. To estimate spinal cord atrophy, the individual normalization to average section size (average of 4 outer rostral levels and 4 caudal levels) was used for each individual rat.

2.4. Statistical analyses

Data were analyzed by analysis of variance (ANOVA) and repeated measures ANOVA followed by post-hoc comparisons (Tukey's test), where appropriate. For missing values (i.e. gait analysis) a mixed model was used followed by post-hoc comparisons (Tukey's test) where appropriate using GraphPad 9 (GraphPad Software, Inc. La Jolla, CA). Differences were statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Effect was considered significant if $p < 0.05$. Data is represented as the mean and standard error to the mean (SEM).

2.5. Data sharing

Study data are deposited at the Open Data Commons for Spinal Cord Injury (ODC-SCI; RRID:SCR_016673) and available for download (<https://odc-sci.org/data/1597>). The ODC-SCI is a secure, cloud-based repository platform designed to share research data (Torres-Espin et al., 2018).

Experimental protocols and detailed methods can be found with the dataset, and any additional information will be provided to researchers upon request.

3. Results

3.1. Animal enrollment, exclusions, and general health monitoring

Animal inclusion and exclusion criteria are summarized in Fig. 1. A total of 92 rats underwent baseline BBB and VF assessments. Animals were required to exhibit a BBB score of 21 and an average baseline PWT (mean of left and right hindpaws) of at least 14 g to qualify for study enrollment. These criteria were established to ensure that only animals without evidence of pre-existing tactile hypersensitivity or baseline locomotor abnormalities were included in the study. The VF criterion was incorporated because a primary objective of the study was to evaluate the development of SCI-induced tactile hypersensitivity. Inclusion of animals with reduced baseline PWT values could increase variability and confound the interpretation of post-injury sensory outcomes.

Following surgery, 8 animals were excluded: 1 due to an anesthesia-related complication, 6 due to injury parameters that did not meet predefined acceptance criteria, and 1 due to surgical complication. This resulted in 76 animals included following surgery, with group sizes of n

= 15 for the Sham, 170 Kdyn, 200 Kdyn, and 250 Kdyn groups, and $n = 16$ for the 250 Kdyn +3 s dwell group.

One animal in the 250 Kdyn +3 s dwell group developed a urinary bladder complication during the first week after SCI and was euthanized according to humane endpoint criteria. Final group sizes were therefore $n = 15$ per group. No additional animals were excluded from the study, and all surviving animals completed all behavioral assessments. For NC analyses, videos with technical acquisition issues were excluded, including one video from the 250 Kdyn +3 s dwell group at Week 3 and four videos at Week 5 (170 Kdyn, $n = 1$; 200 Kdyn, $n = 2$; 250 Kdyn, $n = 1$). In addition, several NC HL paw prints could not be quantified in animals from the 250 Kdyn and 250 Kdyn +3 s dwell groups because these animals lacked weight-supported HL stepping. Consequently, sample sizes varied across specific NC outcome measures and are reported in the corresponding figure legends. All animals were included in the histological analyses.

Rats were closely monitored for bladder function and overall health. Baseline body weight was comparable across groups. After T8 SCI, rats exhibited a transient, severity-dependent reduction in body weight lasting up to 3 weeks. Body weight and bladder expression data are summarized in Supplementary Fig. 1. Sham rats required ≤ 1 day of bladder expression, while the 170, 200, 250, and 250 Kdyn +3 s dwell groups required an average of 6.1, 10.6, 16.2, and 23.3 days, respectively.

3.2. Open field locomotor performance decreased with SCI severity in a graded fashion

Assessment of HL movements using the BBB scale and sub-score as well as the NC gait analysis confirmed that increasing contusive force reduced locomotor performance in the open field starting at Day 1 after T8 SCI (timepoint “0”) that persisted over the course of the experiment (Fig. 2A). BBB testing showed that isolated HL joint movement was rarely observed on Day 1 in SCI groups receiving the most severe injuries, with the 250 Kdyn and 250 Kdyn+3 s dwell groups receiving average BBB scores of 0.23 ± 0.23 (mean $\pm$ SEM) and 0.7 ± 0.22 , respectively. Conversely, rats with milder SCI allocated to 170 and 200 Kdyn groups produced isolated movements of hip, knee and ankle at Day 1, scoring 6.4 ± 0.7 and 3.1 ± 0.7 points, respectively. The BBB scores of the 4 SCI groups were significantly different from each other at every

other timepoint.

The BBB sub-score provides a semi-quantitative scale delineating specific aspects of locomotion including paw position, toe clearance, trunk and tail stability without the confound of FL-HL locomotion. At Day 1 and Week 1 after SCI most rats had a BBB sub-score of 0 (Fig. 2B). Rats allocated to the 250 Kdyn and 250 Kdyn +3 s dwell cohorts did not recover finer aspects of gait over the course of the experiment, averaging 0 and 0.2 ± 0.2 sub-score points at Week 5, respectively. Rats from the 170 and 200 Kdyn cohorts recovered sub-score points at Week 1 had an average BBB sub-score of 6.0 ± 0.62 , and 2.67 ± 0.65 at Week 5, respectively. BBB sub-scores differed significantly among the 170, 200, and 250 Kdyn groups at Week 4 and Week 5 post-SCI. No significant differences were detected between the two most severe injury groups (250 and 250 Kdyn +3 s dwell) at any time point.

Importantly, at 5 weeks post SCI, the average BBB score for each group corresponded to distinct milestones in locomotor recovery—the ability to place the hindpaw with the plantar surface to the open field, the ability to take a few plantar steps, to step consistently or to produce coordinated locomotion, 250 Kdyn +3 s dwell, 250 Kdyn, 200 Kdyn and 170 Kdyn experimental groups, respectively, as shown by others previously (Carter et al., 2016; Park et al., 2016; Scheff et al., 2003). Recovery was most limited in the 250 Kdyn +3 s dwell group, where only 1 of 15 animals recovered weight-supported stepping and 2 additional animals achieved weight-supported standing without stepping. The remaining animals were unable of weight support but 9 of them recovered plantar paw placement without weight-support. Recovery was greater in the 250 Kdyn group, where 11 of 15 animals recovered weight-supported stepping; however, only 2 of those animals demonstrated occasional FL/HL coordination, while the remaining 4 animals achieved plantar placement without weight-supported stepping. In contrast, all animals in the 200 Kdyn group recovered consistent weight-supported stepping, and 9 of 15 exhibited some degree of FL/HL coordination. The greatest functional recovery was observed in the 170 Kdyn group, where all animals demonstrated at least occasional coordination and 5 of 15 achieved consistent coordination with toe clearance. Further details, including comprehensive graphical representations of individual datapoints at each timepoint for open field testing can be found in Supplementary Fig. 2.

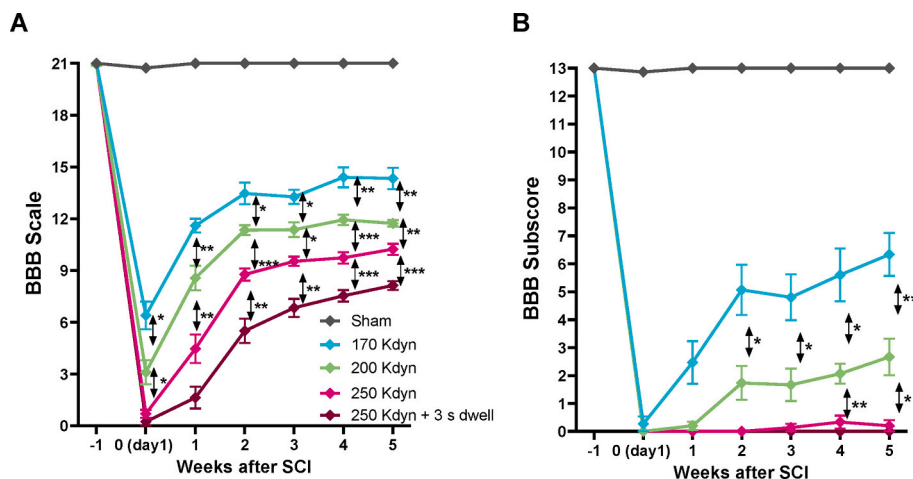


Fig. 2. Graded contusive SCI produced severity-dependent locomotor impairments. (A) Weekly BBB scores over 5 weeks post-injury demonstrating graded recovery according to injury severity. (B) BBB sub-scores indicate minimal recovery of fine locomotor features in severe injuries, whereas milder injuries showed partial improvements. Data are presented as mean $\pm$ SEM; $n = 15$ animals/group. Statistical analysis was performed using two-way repeated-measures ANOVA followed by Tukey's multiple-comparisons test. For BBB scores, significant effects were observed for injury severity ($F(4, 70) = 384.7, p < 0.0001$), time ($F(3.433, 240.3) = 872.7, p < 0.0001$), and injury severity $\times$ time interaction ($F(24, 420) = 65.75, p < 0.0001$). For BBB sub-scores, significant effects were observed for injury severity ($F(4, 70) = 331.9, p < 0.0001$), time ($F(3.900, 273.0) = 697.4, p < 0.0001$), and injury severity $\times$ time interaction ($F(24, 420) = 55.33, p < 0.0001$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Machine learning-assisted analysis of open field locomotion reveals distinct functional groups

Analysis in the NC device revealed the total distance traveled (A), the total time spent locomoting (B) and the average walking speed (C) of rats as depicted in Fig. 3A–C. The 170 Kdyn group displayed a similar overall performance to the Sham group at all timepoints tested and was similar to baseline values for all other groups (Fig. 3). Not surprisingly, the severe SCI groups (250 Kdyn, 250 Kdyn +3 s dwell) traveled the least distance, locomoted for the least amount of time, and had the slowest average “walking” speed of all groups at Week 3 and Week 5. The moderately contused group (200 Kdyn) displayed intermediate recovery of distance traveled, time in motion and walking speed compared to other groups, with values similar to the mild 170 Kdyn group and different from the more severe 250 Kdyn and 250 Kdyn +3 s dwell groups.

Paw print counts captured during the 5-min NC session provided an estimate of step frequency per animal. To account for overall locomotor activity, HL stepping was normalized to FL usage by calculating an HL/FL paw print ratio, defined as the average HL print count divided by the average FL print count for each subject. HL paw print count, and the ratio of HL step to FL step counts revealed that rats in the Sham group or SCI rats at baseline took more HL steps than FL steps over the 5-min assessment in the NC and that the ratio of HL steps to FL steps decreased as SCI severity increased (Fig. 4) indicating increased FL reliance after injury. Both HL paw print counts (Fig. 4B) and HL/FL ratios (Fig. 4C) differed significantly across most SCI groups, demonstrating sensitivity to graded locomotor deficits. Moreover, measurements of forepaw and hindpaw print area, diameter and intensity revealed differences with respect to SCI severity (Supplemental Fig. 3). Compared to the Sham group, hindpaw prints of SCI rats had smaller print area, reduced diameter and lower intensity. In contrast, FL paw prints of SCI rats had larger print area, increased diameter and higher intensity compared to the Sham group, consistent with a shift in weight support from HL to FL.

Importantly, the absence of weight-supported steps in most rats assigned to the 250 Kdyn and 250 Kdyn+3 s dwell groups resulted in a reduced sample size for the HL gait geometry and dynamics data, with only $n = 7$ –10 for the 250 Kdyn group and $n = 1$ –2 for the 250 Kdyn+3 s dwell group included in analysis. Additionally, some gait geometry and dynamic gait parameters are reported below from averages of stepping bouts at speeds of less than 30 cm/s (low speed) that all rats with the ability to step could achieve.

3.4. Unbiased analysis of gait metrics identify specific locomotor phenotypes

FL and HL base of support widen after SCI in a severity dependent manner (Fig. 5A–B). NeruoCube analysis at Week 5, showed that the 200 Kdyn, 250 Kdyn and 250 Kdyn +3 s dwell groups exhibit significantly wider FL base of support than Sham and mild (170 Kdyn) SCI groups, while HL base of support increases with increased SCI severity, among animals with quantifiable HL stepping. In addition, SCI rats' forepaw placement relative to their center of mass was shifted rostrally, while hindpaw placement was shifted caudally compared to Sham rats and baseline values (Supplemental Fig. 4). Notably, the forepaw position shift was graded, with values increasing according to SCI severity and showing significant differences between SCI cohorts. In contrast, the HL paw position differs significantly from Sham but does not follow a graded pattern. This elongation and widening of the rat's base of support with increased SCI severity may serve to stabilize the rat during movement and stance.

Interestingly, HL stride length for low-speed steps (<30 cm/s) was slightly longer than Sham reaching significance only for the 200 Kdyn group at Week 3 and 5 after SCI and 250 Kdyn group at Week 3 after SCI (Fig. 5D). However, FL stride length was shorter in all SCI groups

compared to the Sham group or their pre-injury values (Fig. 5C). The 170 Kdyn and 200 Kdyn groups had similar FL stride lengths, and the 250 Kdyn and 250 Kdyn +3 s dwell groups were also similar to one another at both Week 3 and Week 5. The proportion of time a rat is in weight supported stance in the FL or HL during each step cycle or duty cycle is displayed in Fig. 5E–F. Compared to baseline and Sham rats, SCI rats showed increased duty cycle ratios in the FL and decreased HL duty cycle as SCI severity increased. The average duration of swing phases of the step cycle is reduced in FLs of all SCI groups compared to the Sham group, but they are not different from one another. In the HL, as SCI severity increases, the average duration of swing phase of gait increases and the stance duration decreases (Supplemental Fig. 5B, D).

3.5. Unbiased gait analysis reveals disruption of coordinated step patterns in SCI

Interlimb coordination patterns were automatically classified by the NC platform. Three patterns were identified: alternating (limb movements alternate between left and right, e.g., RF–RH–LF–LH), cruciate (limbs on the same side move in sequence, e.g., RF–LF–RH–LH), and rotatory (limbs progress in a circular sequence, e.g., RF–LF–LH–RH) as described previously (Cheng et al., 1997; Hamers et al., 2001). The ratio of gait cycles for each respective pattern were provided by the NC platform for each paw individually. The data represents the percentage of step cycles that a paw is engaged in a particular step pattern. At baseline and in Sham-operated animals, the percent of step patterns calculated for HL and FL showed similar proportions, with most steps following a predominant alternating pattern ($28.8 \pm 0.86\%$), followed by a cruciate pattern ($19.2 \pm 0.56\%$), and a smaller proportion of rotatory steps ($2.8 \pm 0.23\%$) (Fig. 6). After SCI, rats showed a significant decrease in alternating and cruciate step patterns, with reductions following a graded pattern according to injury severity. In contrast, the frequency of rotatory step patterns increased, though not in a graded manner. The total percentage of coordinated step patterns (alternating + cruciate + rotatory) decreased with increasing injury severity. This data set shows significant differences between Sham as well as between lesion severities, indicating that while some rats retain the ability to step after SCI, their stepping sequence and coordination are substantially disrupted compared to Sham.

3.6. Sensorimotor function decreased with SCI severity

Fine locomotor performance was evaluated by calculating the percentage of HL and FL missteps or error rate during the horizontal ladder test. To ensure inclusion of all animals, including those with severe SCI that were unable to generate HL plantar steps, rats were assigned an error rate of 100%, reflecting complete failure to advance the HL and support body weight during the task.

No experimental group demonstrated significant increase in FL errors on the ladder task at any timepoint (Supplementary Fig. 6). However, increased SCI severity significantly increased the HL error rate at Weeks 3 and 5 after SCI (Fig. 7). Sham SCI rats at baseline exhibited very few HL placement errors ($2.85 \pm 0.51\%$). After SCI, rats allocated to the 250 Kdyn+3 s dwell group presented the highest HL error scoring 90.78 ± 6.30 and 94.53 ± 3.0 at 3 and 5 Weeks, respectively. In contrast, rats receiving a less severe, 170 Kdyn SCI take fewer missteps with the HLs as evidenced by a lower HL error scoring, $26.33 \pm 3.92\%$ and $22.41 \pm 2\%$ at Week 3 and Week 5, respectively. These data demonstrate significant differences between Sham and between injury severity groups for sensorimotor function as assessed by the horizontal ladder test.

3.7. Hindpaw neuropathic pain increased with SCI severity

Hindpaw hypersensitivity to a mechanical stimulus was assessed using the von Frey test preoperatively and a Week 3 and Week 5 post-SCI. For each animal, the lowest PWT from either hindpaw was used

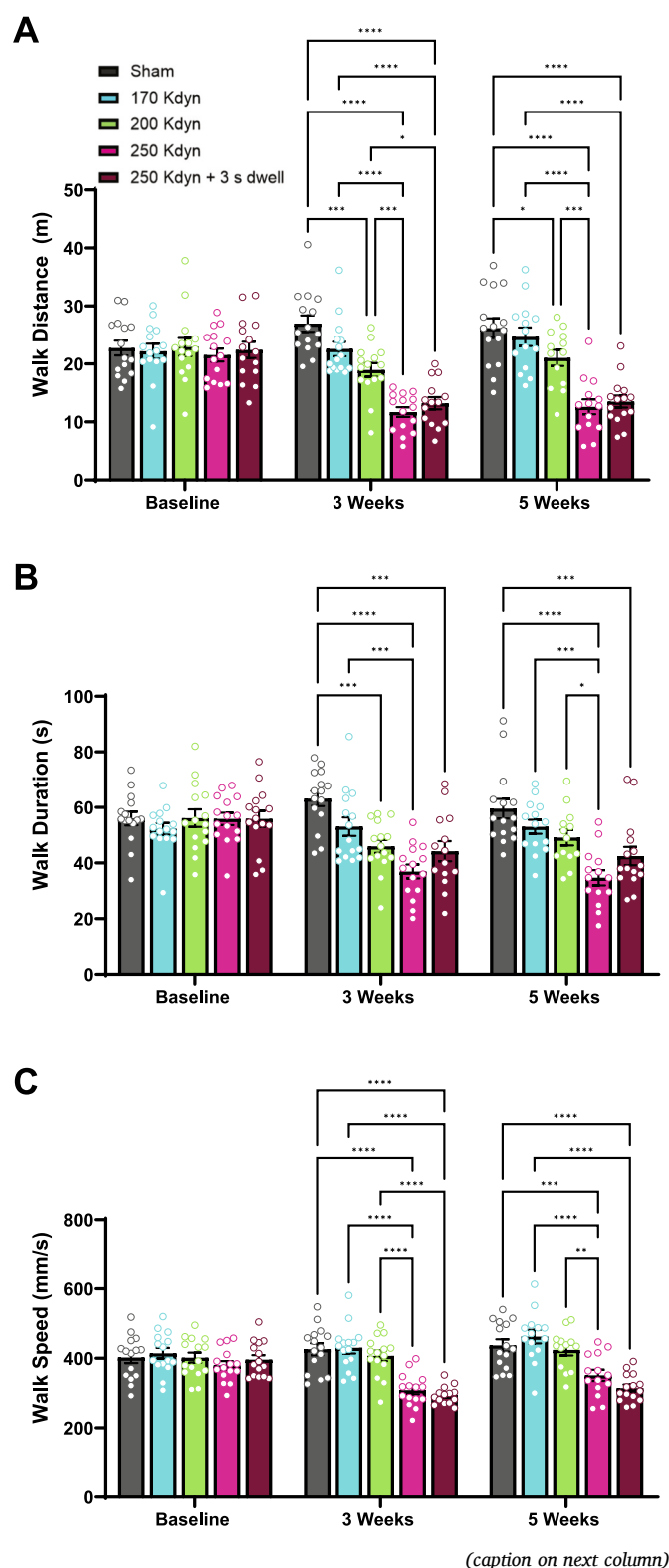


Fig. 3. Increasing SCI severity reduces spontaneous locomotor activity and walking speed. (A) Total distance traveled decreased with injury severity. (B) Walk duration decreased with increasing injury severity. (C) Average walking speed remained comparable to Sham for mild injuries (170 and 200 Kdyn) but decreased in severe injuries (250 Kdyn, 250 Kdyn + 3 s dwell) at Week 3 and 5 after SCI. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. For total distance traveled, significant effects were observed for injury severity ($F(4, 70) = 17.98, p < 0.0001$), time ($F(2, 135) = 18.13, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 12.21, p < 0.0001$). For walk duration, significant effects were observed for injury severity ($F(4, 70) = 8.497, p < 0.0001$), time ($F(2, 135) = 16.67, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 7.142, p < 0.0001$). For average walking speed, significant effects were observed for injury severity ($F(4, 70) = 15.82, p < 0.0001$), time ($F(2, 135) = 9.341, p = 0.0002$), and injury severity $\times$ time interaction ($F(8, 135) = 8.932, p < 0.0001$). $n = 15$ animals/group except at Week 3 (250 Kdyn + 3 s dwell, $n = 14$) and Week 5 (170 Kdyn, $n = 14$; 200 Kdyn, $n = 13$; 250 Kdyn, $n = 14$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

to represent mechanical allodynia, reflecting the most sensitive site rather than averaging left and right values (Fig. 8A). Sham-lesioned animals exhibited no change in paw withdrawal threshold at 3- and 5-weeks post-SCI. Conversely, all 4 SCI cohorts showed a significant decrease in PWT, with more pronounced reductions observed in the 250 Kdyn and 250 Kdyn+3 s dwell cohorts. Some animals did not exhibit a significant decrease in PWT compared to baseline values, maintaining PWT values of 13 g or higher. Specifically, at 3 weeks post-SCI, this was observed in 6, 4, 7, and 3 out of 15 animals for the 170, 200, 250, and 250 Kdyn+3 s dwell groups, respectively. By 5 weeks, these numbers decreased to only 2, 3, 1, and 1 rat showing no changes in von Frey values for the 170, 200, 250, and 250 Kdyn+3 s dwell groups, respectively.

Hindpaw hypersensitivity to cold stimulus was evaluated using the Acetone test at Week 5 after SCI. Each hindpaw (left and right) was stimulated 5 times, and the total number of aversive responses for each paw is presented (combined left and right paw values, $n = 30$ /group; Fig. 8B). Sham-lesioned rats reacted to acetone exposure between 0 and 3 times, with an average of 0.86 ± 0.25 reactions. Rats allocated to SCI lesion presented increased reactivity showing an average of 1.53 ± 0.27 , 2.0 ± 0.27 , 4.93 ± 0.7 and 6.67 ± 0.5 responses for the 170 Kdyn, 200 Kdyn, 250 Kdyn and 250 Kdyn+3 s dwell groups, respectively. Significant differences between Sham and 250 Kdyn and 250 Kdyn+3 s dwell cohorts, but not between Sham and the milder SCI lesions were found.

Although the Hargreaves test has been reported as sensitive in thoracic compression and contusion SCI models, (Berrocal et al., 2014; Dugan and Sagen, 2015; Hama and Sagen, 2007; Jergova et al., 2023) hindpaw responses to heat in our study did not differ significantly from baseline or Sham controls (Supplementary Fig. 7).

3.8. Graded SCI produces progressive tissue loss and reduced spared spinal cord tissue

Histological analysis of a ~ 3 cm segment of the spinal cord containing the T8 lesion epicenter revealed graded tissue loss across SCI groups. Fig. 9 shows the average areas of spared tissue (A) and lesion (C) across sectioning planes from rostral to caudal, with the lesion epicenter designated as level 0 as revealed by myelin staining.

All SCI groups exhibited reduced total spinal cord area compared to Sham, including in segments distal to the epicenter. Lesioned tissue was detected in all SCI samples up to 1.4 cm rostral to the epicenter, primarily affecting the dorsal columns. Caudal to the epicenter, lesion extent in milder SCI cohorts (170 and 200 Kdyn) reached up to 5 mm, whereas severe lesions (250 Kdyn, 250 + 3 Kdyn) extended up to 12 mm.

Volume calculations confirmed a graded reduction in total spinal cord volume with increasing injury severity (Fig. 10A). Lesion volume increased progressively, with significant differences between all SCI

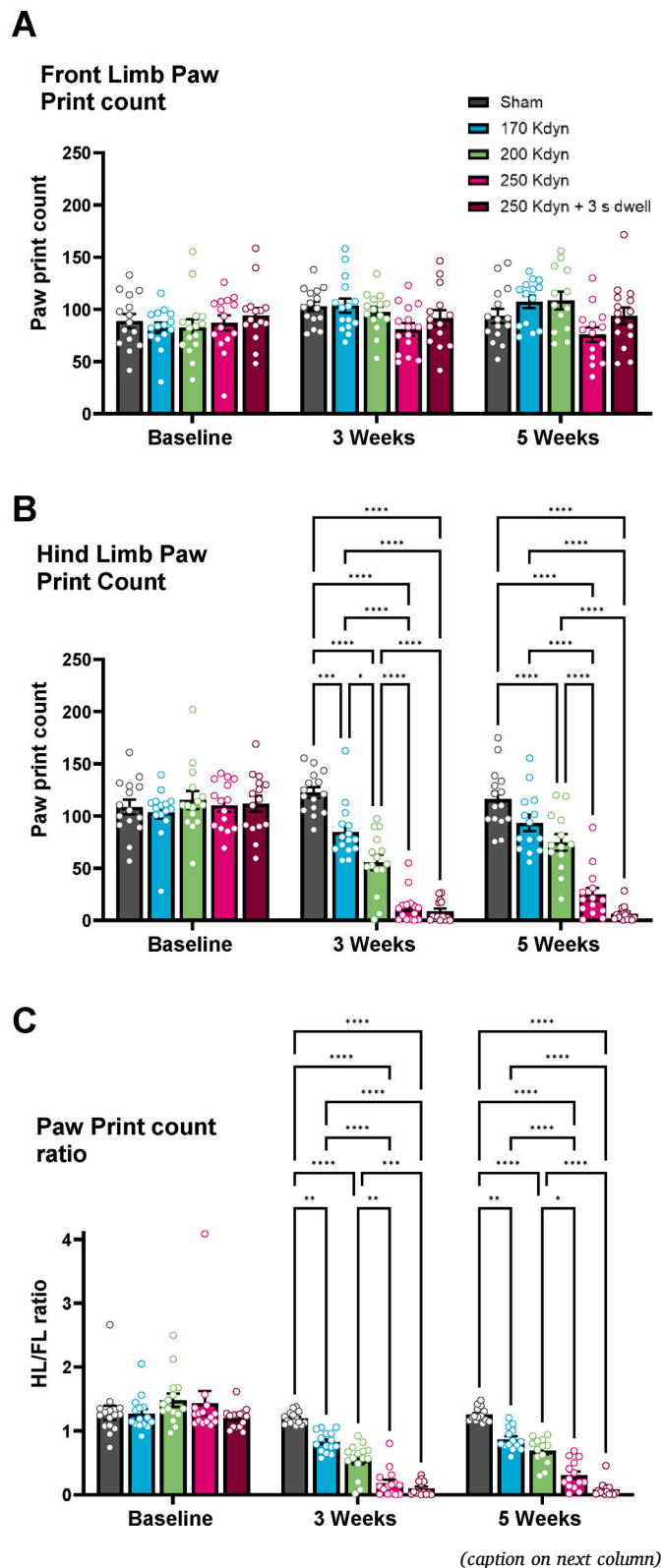


Fig. 4. Hindlimb (HL) paw print count and HL/Forelimb (FL) ratio decreased according to SCI severity (A-B) FL and HL paw print counts during the 5 min NeuroCube test. After SCI, HL paw print counts decreased in a severity dependent manner, while FL print counts remained comparable between groups. (C) HL/FL paw print ratio revealed significant differences between all SCI groups except 170 and 200 Kdyn. This ratio strongly correlated with lesion size and BBB scores, supporting its utility as a quantitative measure of locomotor impairment. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. For FL paw print count, no significant effect of injury severity was observed ($F(4, 70) = 1.892, p = 0.1215$), whereas significant effects were observed for time ($F(2, 135) = 3.665, p = 0.0282$) and injury severity $\times$ time interaction ($F(8, 135) = 2.228, p = 0.0290$). For HL paw print count, significant effects were observed for injury severity ($F(4, 70) = 48.34, p < 0.0001$), time ($F(2, 135) = 129.7, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 25.55, p < 0.0001$). For the HL/FL ratio, significant effects were observed for injury severity ($F(4, 70) = 32.72, p < 0.0001$), time ($F(2, 135) = 195.1, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 17.06, p < 0.0001$). $n = 15$ animals/group except at Week 3 (250 Kdyn + 3 s dwell, $n = 14$) and Week 5 (170 Kdyn, $n = 14$; 200 Kdyn, $n = 13$; 250 Kdyn, $n = 14$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

groups except 170 versus 200 Kdyn (Fig. 10B). Spared tissue volume decreased proportionally to injury severity, with differences noted across all groups except 250 versus 250 Kdyn+3 s dwell (Fig. 10C). The percentage of spinal cord area occupied by lesion (Fig. 10D) revealed the most pronounced differences between SCI cohorts and proved the most sensitive histological measure for distinguishing graded injury severity.

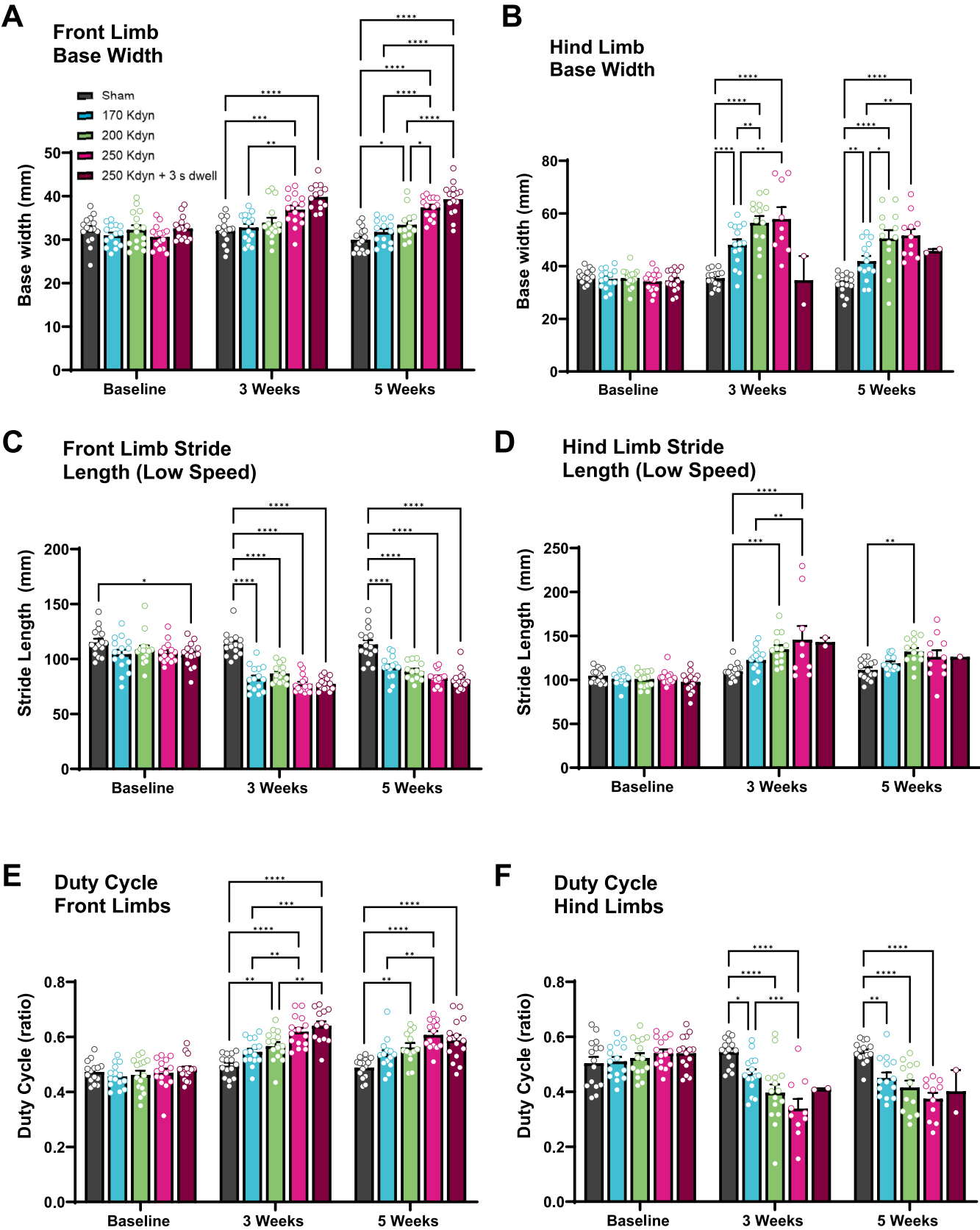
To evaluate the sensitivity of behavior parameter to detect changes in a graded lesion model, each parameter, including locomotor and sensory outcomes, obtained at 5 weeks after SCI was compared with the respective lesion percent area using a Pearson correlation, significant parameters ($p < 0.05$) are listed in Fig. 11, including the R^2 values for each correlation. The highest correlation coefficients were detected for paw count ratio (Fig. 11A), BBB score (Fig. 11B), Ladder test error percent for HL (Fig. 11C), and FL stride length (All Speeds, Fig. 11D). It is interesting to note that several parameters for FL gait data presented some of the strongest correlations, with the FL Stride Length showing a R value of 0.82.

4. Discussion

One of the more underappreciated challenges in SCI drug development is that the injury model, intervention timing, and outcome measures must all be deliberately matched to the intervention's perceived mechanism of action, and a mismatch on any one of these factors may cause an effective therapy to appear ineffective. This contrasts drug development efforts in other fields like epilepsy and pain, where a compound either suppresses seizures or reduces a pain response, and the in vivo platforms are built around detecting that effect. In SCI, there is no single readout that is analogous to seizure frequency or pain threshold that can capture therapeutic benefit as SCI affects motor, sensory, and autonomic function simultaneously. The well-characterized and consistent behavioral and histological SCI only data presented in this manuscript may also serve as historical controls for future studies that employ the same injury model and outcome measures, reducing the need for additional control cohorts (or reducing the number needed to achieve statistical power) and supporting the 3Rs principle of animal use reduction. This approach is consistent with ARRIVE and NIH guidelines supporting the use of well-validated historical control data when the injury model and outcome measures are standardized across studies.

4.1. Choosing the right SCI model is imperative for translational success

Injury severity and level of SCI alone introduce meaningful



(caption on next page)

Fig. 5. Graded SCI induces distinct alterations in gait geometry and limb dynamics. (A–B) Forelimb (FL) and Hindlimb (HL) base of support widen in a severity dependent manner after SCI. (C) FL stride length decreased in all SCI cohorts, whereas (D) HL stride length remained largely unchanged. (E–F) Duty cycle ratios increased in FLs and decreased in HLs with increasing injury severity. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. For FL base of support, significant effects were observed for injury severity ($F(4, 70) = 13.03, p < 0.0001$), time ($F(2, 135) = 40.77, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 11.85, p < 0.0001$). For HL base of support, significant effects were observed for injury severity ($F(4, 70) = 15.36, p < 0.0001$), time ($F(2, 100) = 54.29, p < 0.0001$), and interaction ($F(8, 100) = 15.35, p < 0.0001$). For FL stride length, significant effects were observed for injury severity ($F(4, 70) = 34.25, p < 0.0001$), time ($F(2, 135) = 78.22, p < 0.0001$), and interaction ($F(8, 135) = 4.709, p < 0.0001$). For HL stride length, significant effects were observed for injury severity ($F(4, 70) = 5.548, p = 0.0006$), time ($F(2, 99) = 47.53, p < 0.0001$), and interaction ($F(8, 99) = 3.831, p = 0.0006$). For FL duty cycle ratio, significant effects were observed for injury severity ($F(4, 70) = 9.767, p < 0.0001$), time ($F(2, 134) = 163.6, p < 0.0001$), and interaction ($F(8, 134) = 9.075, p < 0.0001$). For HL duty cycle ratio, significant effects were observed for injury severity ($F(4, 70) = 8.929, p < 0.0001$), time ($F(2, 102) = 30.85, p < 0.0001$), and interaction ($F(8, 102) = 8.083, p < 0.0001$). For FL analyses, $n = 15$ animals/group except at Week 3 (250 Kdyn +3 s dwell, $n = 14$) and Week 5 (170 Kdyn, $n = 14$; 200 Kdyn, $n = 13$; 250 Kdyn, $n = 14$). For HL analyses, $n = 15$ animals/group except at Week 3 (200 Kdyn, $n = 14$; 250 Kdyn, $n = 9$; 250 Kdyn +3 s dwell, $n = 2$) and Week 5 (170 Kdyn, $n = 14$; 200 Kdyn, $n = 13$; 250 Kdyn, $n = 11$; 250 Kdyn +3 s dwell, $n = 2$). Reduced HL sample sizes reflect the inability to quantify gait parameters in animals lacking consistent weight-supported stepping. Post-hoc comparisons for the 250 Kdyn +3 s dwell HL cohort are not shown because the limited sample included only animals with sufficient weight-supported stepping and was not representative of the full group. Significant Tukey's post-hoc comparisons for the remaining groups are indicated in the figure; adjusted p -values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

complexity to therapeutic development efforts. For example, potential neuroprotective agents initiated acutely within 12 h of SCI might be best examined in a severe injury model, where sparing a small amount of white matter could elicit meaningful functional gain, with recovery tracked periodically to identify changes in locomotor patterns (e.g., weekly BBB scoring) (Kloos et al., 2005; Schucht et al., 2002). Conversely, interventions that drive adaptive, beneficial plasticity or reduce aberrant plasticity, or encourage regeneration may require a moderate injury (i.e., 170–200 Kdyn) where sufficient spared tissue remains to support neural reorganization. Thoracic injuries are the most commonly modeled in pre-clinical studies and primarily affect trunk stability and HL function, while cervical injuries additionally compromise FL and hand function, respiratory capacity, and involve distinct spinal circuitry (Fouad et al., 2020; Wilcox et al., 2017). This distinction matters therapeutically: an intervention that improves HL stepping after thoracic injury may have limited relevance for restoring hand function after cervical injury. Conversely, interventions geared toward improving truncal control and balance may be better suited to be tested in a thoracic contusion rather than a cervical one. Lumbar injuries, though less common clinically, involve significant grey matter loss, including lower motor neuron populations and locomotor-related interneurons and present yet another biological context that may respond differently to the same intervention. (Magnuson et al., 2005; Patel et al., 2014) Model selection must therefore account not only for severity, but for whether the injury level and resulting functional deficits are appropriate for the intended clinical population. In the current platform, we employed a mid-thoracic contusion model as an established and well-characterized starting point; a cervical contusion model is currently being finalized and will expand the platform's utility for the growing proportion of investigators focused on cervical SCI.

The natural recovery pattern following thoracic contusive SCI consists of profound motor deficits during the first few days after injury that gradually improve over time, with the degree of impairment depending on injury severity. Our findings are consistent with previous reports using the IH impactor, which also demonstrated differences in BBB scores among contusion severities as early as Days 1–2 post-injury (Carter et al., 2016; Scheff et al., 2003). In the present study, animals in the mildest contusion group exhibited non-zero BBB scores during this period; however, these animals displayed only occasional isolated hindlimb joint movements during the 4-min observation period and did not exhibit plantar stepping. Thus, the early non-zero BBB scores should not be interpreted as meaningful locomotor recovery but rather as subtle differences in the severity of the initial injury.

Another layer of complexity comes in the form of lesion type. An intervention targeting the corticospinal tract (CST) tested in a contusion model rather than a dorsal column lesion that specifically disrupts the CST risks masking a real effect because intact reticulospinal and rubrospinal pathways can drive recovery independent of the treatment (Bieler et al., 2018; Fouad et al., 2021; Schucht et al., 2002). Further,

outcome measure selection in a thoracic SCI therapeutic screening platform must be guided by all the above previously described factors. The BBB locomotor scale, while widely used, reflects recovery driven largely by activation of lumbar locomotor circuitry from reticulospinal and propriospinal pathways and any adaptive reorganization that occurs below the injury, indicating that an intervention that successfully promotes CST regeneration could show no BBB improvement whatsoever while producing robust gains on a horizontal ladder. Intervention timing also matters, since the lesion environment evolves between the acute and chronic phases of injury. An agent with a narrow therapeutic window for neuroprotection may produce a negative result simply because it was administered too late.

It is important to note that although CROs that conduct standardized, consistent behavioral testing may address some barriers in translation, it is critical to understand and characterize a potential therapeutic prior to behavioral testing. For example, researchers must understand the mechanism of action of the therapeutic, conduct pharmacokinetics such as bioavailability and central nervous system penetration, ensure the therapeutic interacts with and modifies the intended target, define the pharmacodynamic time course of target engagement of target engagement (e.g., receptor occupancy), and conduct dose-range finding studies to determine the ideal dose and window for behavior.

Other considerations can also shape the ability to detect therapeutic efficacy, like the length of the study. Review of the longitudinal data collected in this experiment revealed that the natural behavioral recovery may not be complete. This indicates that survival duration post SCI and/or therapeutic could be adjusted based on anticipated therapeutic action or examination of the recovery curves at the end of 5 weeks. Additionally, buprenorphine or other analgesics (i.e. acetaminophen, ibuprofen) are administered in some experimental studies to combat post-surgical pain, but the dosing strategy used in experimental SCI does not represent the multitude of analgesia or the polypharmacy prescribed in the clinic (Kitzman et al., 2017). For reparative treatments, combining an intervention with rehabilitative training may be essential to translating enhanced neuronal growth into functional recovery (Sachdeva et al., 2016; Theisen et al., 2017; Torres-Espin et al., 2018). Importantly, while beyond the scope of this experiment, future work will implement aerobic or strength training rehabilitative strategies or with increased home cage activity in conjunction with therapeutics (Chariker et al., 2020; Chhaya et al., 2019; Giddings and Detloff, 2026; Khodaparast et al., 2016). Linking anatomical evidence of regeneration, regrowth, or plasticity to specific behavioral improvements remains challenging, but this process begins with selecting outcome measures appropriate for the lesion type. Data presented here demonstrate the sensitivity of the selected locomotor and sensory tests and histological measures in detecting variations in spinal cord lesion severity and respective functional recovery. Importantly, these data align with similar work in the field (Basso et al., 1996a; Basso et al., 1996b; Fedorova et al., 2022; Kelley et al., 2014; Kloos et al., 2005; Krizsan-

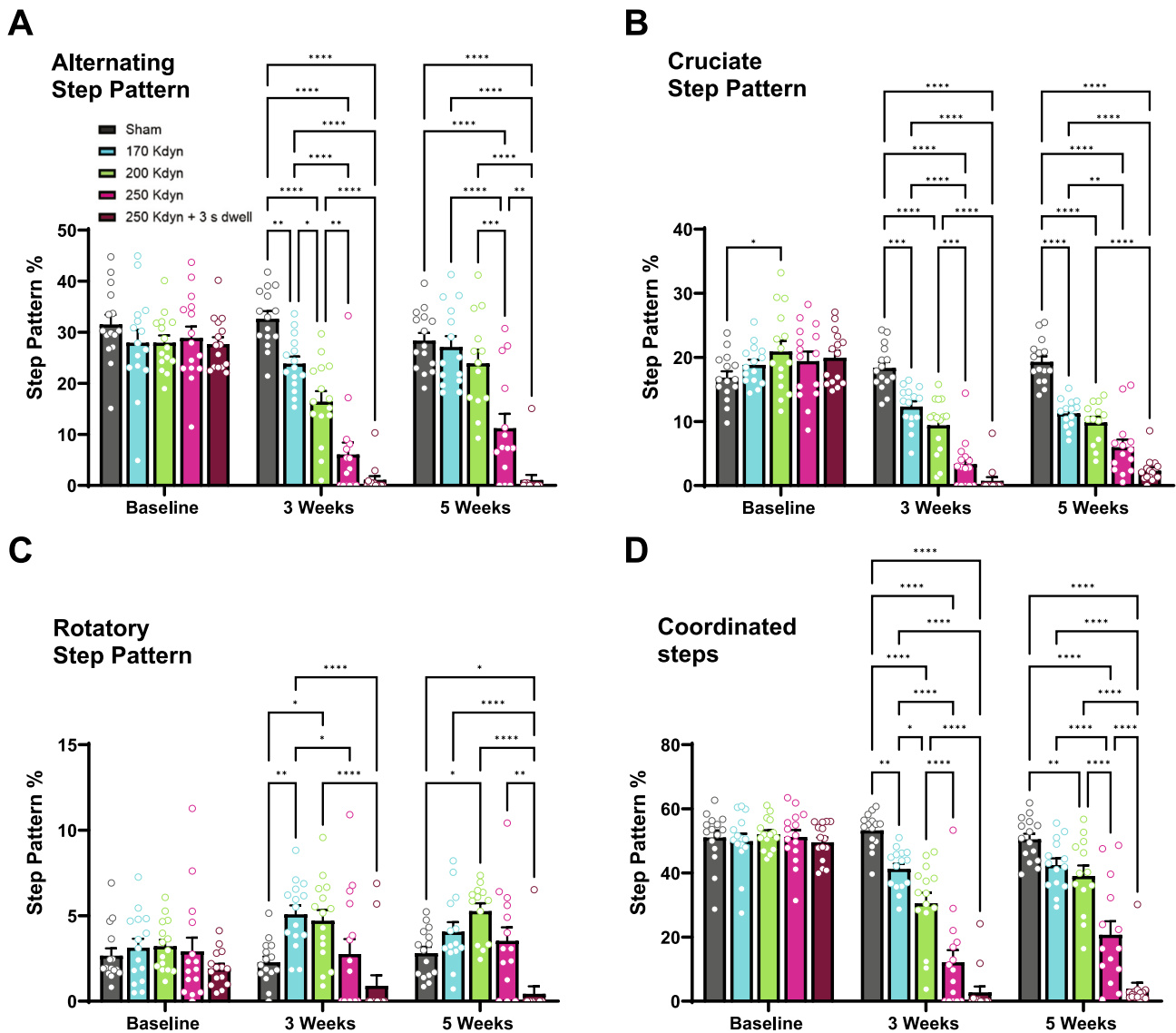


Fig. 6. Increasing SCI severity disrupts interlimb coordination and promotes uncoordinated gait patterns. Proportion of alternating (A), cruciate (B), or rotatory (C) steps were quantified by the NeuroCube platform. Total proportion of coordinated steps (alternating + cruciate + rotatory) is shown in (D). Following SCI, the percentage of coordinated steps decreased in a severity-dependent manner. Alternating and cruciate patterns declined, whereas rotatory steps increased. Severe SCI groups (250 and 250 Kdyn + 3 s dwell) exhibited more than 80% uncoordinated steps by Week 5 after SCI. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a mixed-effects model followed by Tukey's multiple-comparisons test. For alternating steps, significant effects were observed for injury severity ($F(4, 70) = 51.68, p < 0.0001$), time ($F(2, 135) = 69.22, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 135) = 14.23, p < 0.0001$). For cruciate steps, significant effects were observed for injury severity ($F(4, 70) = 42.02, p < 0.0001$), time ($F(2, 135) = 175.4, p < 0.0001$), and interaction ($F(8, 135) = 22.60, p < 0.0001$). For rotatory steps, significant effects were observed for injury severity ($F(4, 70) = 15.36, p < 0.0001$) and interaction ($F(8, 135) = 2.178, p = 0.0329$), but not time ($F(2, 135) = 0.9674, p = 0.3827$). For total coordinated steps, significant effects were observed for injury severity ($F(4, 70) = 67.15, p < 0.0001$), time ($F(2, 135) = 151.7, p < 0.0001$), and interaction ($F(8, 135) = 26.08, p < 0.0001$). $n = 15$ animals/group except at Week 3 (250 Kdyn + 3 s dwell, $n = 14$) and Week 5 (170 Kdyn, $n = 14$; 200 Kdyn, $n = 13$; 250 Kdyn, $n = 14$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Agbas et al., 2014) and demonstrate the potential utility of automated gait analysis platforms such as NC for evaluating changes following interventions in clinically relevant rat thoracic spinal contusion models.

4.2. Increased granularity provided by automated open field gait may reveal functional gains of therapeutics

Since its inception in 1995, the BBB locomotor rating scale (Basso et al., 1995) has been the gold standard for SCI research in rats due to its affordability, ease of use and minimal training requirements, as well as the generalizability across research labs. Though extensively validated (Basso et al., 1995, 1996a; Basso et al., 1996b), evaluating

locomotor changes with BBB can be challenging and prone to human error. Accurate assessment of FL-HL coordination (necessary for scores above 12) depends heavily on rater expertise and sufficient animal activity (requiring at least three consecutive steps multiple times). Kloos et al. (2005) showed that while experimenters rated rats with 3 distinctive spinal cord contusions as being coordinated (BBB > 12), a more quantitative gait analysis revealed differences in their coordinative step pattern. In our study, the NC complemented these gaps by revealing that deficits in locomotion after contusive SCI are also characterized by the enhanced use and reliance of FLs, including a forward shift in weight support, shorter and faster FL stepping and wider base of support. Several automatic gait analysis systems are used in preclinical

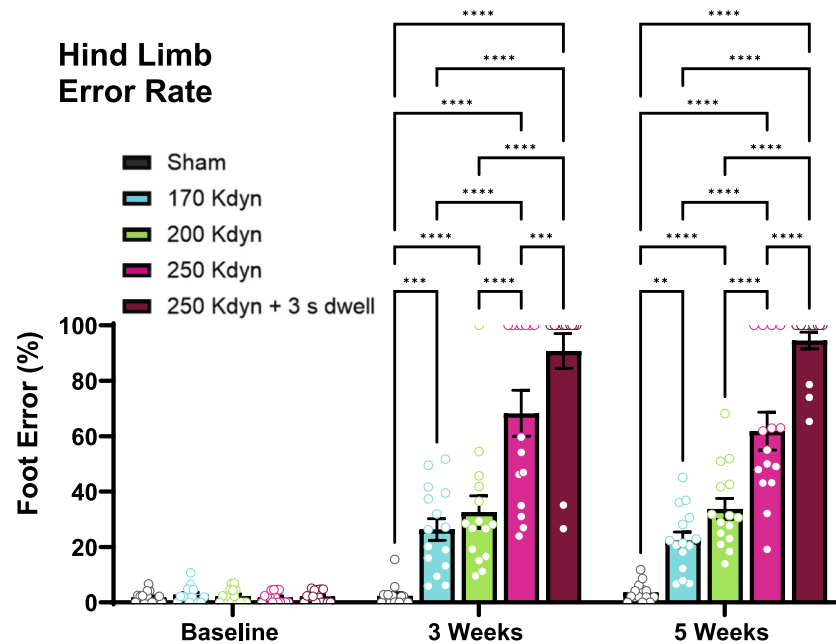


Fig. 7. Hindlimb sensorimotor deficits worsen progressively with increasing SCI severity. Percent Hindlimb (HL) errors during traversal of an irregularly spaced horizontal ladder were measured at baseline, Week 3, and Week 5 post-SCI. HL performance decreased in a severity dependent manner, with higher error rates observed in more severe injury groups. Data are presented as mean $\pm$ SEM. $n = 15$ animals/group. Statistical analysis was performed using two-way repeated-measures ANOVA followed by Tukey's multiple-comparisons test. Significant effects were observed for injury severity ($F(4, 70) = 69.18, p < 0.0001$), time ($F(2, 140) = 239.4, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 140) = 35.14, p < 0.0001$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

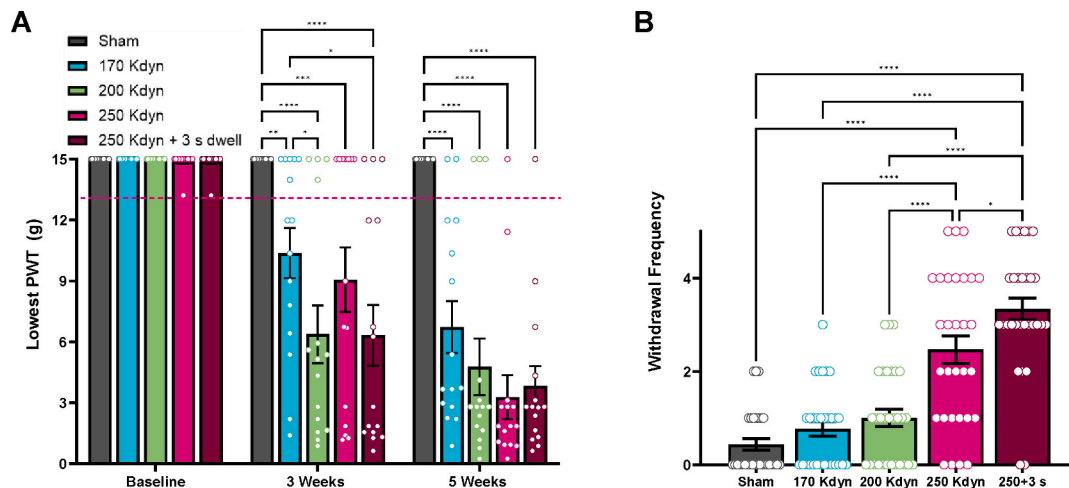


Fig. 8. Severe SCI produces persistent mechanical and cold hypersensitivity. (A) Mechanical allodynia assessed using von Frey filaments. The lowest PWT of either hindpaw was used per animal. SCI reduced PWT in a severity-dependent manner, with severe groups most affected. A subset of animals in each group maintained PWT values ≥ 13 g after SCI (dotted line), comparable to baseline values, after SCI. (B) Cold allodynia assessed using the acetone test. Each hindpaw was stimulated five times, and the number of withdrawal responses for each paw was analyzed as an individual observation. SCI animals exhibited increased aversive responses compared with Sham, with significant differences in the 250 Kdyn and 250 Kdyn + 3 s dwell groups. Data are presented as mean $\pm$ SEM. For mechanical allodynia, $n = 15$ animals/group. Two-way repeated-measures ANOVA showed significant effects of injury severity ($F(4, 70) = 16.53, p < 0.0001$), time ($F(2, 140) = 122.0, p < 0.0001$), and injury severity $\times$ time interaction ($F(8, 140) = 9.250, p < 0.0001$), followed by Tukey's multiple-comparisons test. For cold allodynia, $n = 30$ paw observations/group (two hindpaws/animal). One-way ANOVA showed a significant group effect ($F(4, 145) = 35.53, p < 0.0001$), followed by Tukey's multiple-comparisons test. Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

SCI research, our findings with NC are consistent with previous studies (Datto et al., 2015; Garcia-Ovejero et al., 2014; Hamers et al., 2001; Singh et al., 2011; Timotius et al., 2021; Xing et al., 2022; Zheng et al., 2023). Although gait changes in SCI have been reported with other automated systems, the NC assay differs in key methodological aspects. Importantly, it records spontaneous locomotion as rats walk freely for 5 min. This provides a large number of step cycles to analyze while

reflecting natural speed and activity. For example, CatWalk requires training and typically requires at least 3 runs to result in 9–15 paw prints per rat (Datto et al., 2015; Koopmans et al., 2005; Zheng et al., 2023), whereas the NC consistently captures ~ 80 steps per run in SCI rats. The larger number of step cycles allows analysis across a wider range of speeds, improving sensitivity for detecting subtle locomotor changes. Similar to other publications, we found perturbations in gait either

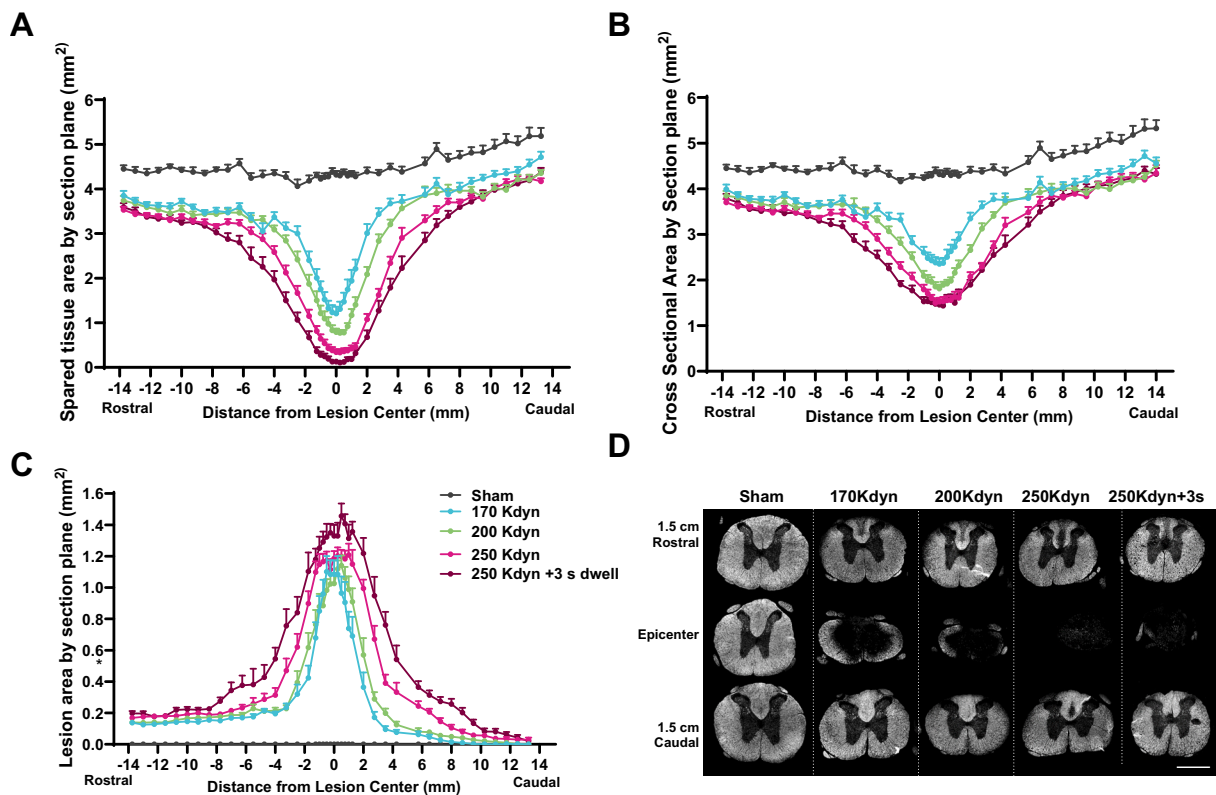


Fig. 9. Increasing SCI severity expands lesion extent and reduces spared spinal cord tissue along the rostro-caudal axis. (A) Spared tissue area at 250 μ m intervals through the rostrocaudal extent of the injury site. SCI groups showed significant reductions compared to Sham, with graded differences across injury severities. (B) Cross-sectional area by distance from epicenter. All SCI groups exhibited significant reductions, including at the most rostral and caudal tissue sections evaluated, reflecting tissue atrophy. (C) Lesion area measured across sections along the rostro-caudal axis. No lesion tissue was detected beyond 14 mm caudal to the epicenter, whereas rostral spread extended to the same distance, primarily affecting the dorsal columns. Lesion area increased progressively with injury severity. (D) Representative spinal cord sections at rostral, epicenter, and caudal levels for each experimental group. Note the white matter lesion extending into the dorsal columns in rostral sections, as well as the corticospinal tract white matter damage observed in the severe injury groups at caudal levels, scale bar represents 1 mm. Data are presented as mean $\pm$ SEM, $n = 15$ samples/group. Data in panels A–C are presented descriptively for graphical representation of the rostro-caudal distribution of tissue and lesion measures; no inferential statistical comparisons were performed.

persisted or became stronger and more significant, when gait parameters were analyzed at different walking speeds (Krizsan-Agbas et al., 2014). Spontaneous in-cage activity is an important correlate of locomotor recovery after SCI (Burke et al., 2023; Caudle et al., 2011) and can be meaningfully altered by therapeutic interventions; as such, it represents a valuable parameter captured by the NC platform. Although assays such as open field exploration (Gazard et al., 2025; Kraeuter et al., 2019; Mills et al., 2001) and the elevated plus maze (Walf and Frye, 2007; Wilcox et al., 2017) could provide complementary insight into spontaneous activity and anxiolytic behaviors, the NC assay offers a straightforward and sufficient measure for the current screening platform; future iterations could incorporate such measures to further strengthen the behavioral battery.

4.3. Multimodal sensory testing may reveal context-dependent analgesia of therapeutics

Neuropathic pain remains a significant concern for people living with SCI, therefore there is an urgent need for novel interventions that alleviate pain associated with SCI. (Anderson, 2004). Approaches aimed at restoring locomotor function, may have neutral, beneficial or even detrimental effects on neuropathic pain. Given the potential for side effects like allodynia and hyperalgesia (Hofstetter et al., 2005), the evaluation of sensory changes is essential for preclinical therapeutic screening and safety profiling. Using validated and widely used methods in the SCI and pain fields, we assessed mechanical and thermal pain modalities across several severities of contusive T8 SCI (Detloff et al.,

2010). Importantly, our data align with previous studies, showing increased mechanical and cold allodynia following SCI, with more severe lesions leading to a more pronounced and consistent hypersensitivity (Detloff et al., 2010; Gaudet et al., 2017; Hama and Sagen, 2007; Kloos et al., 2005; Lindsey et al., 2000; van Gorp et al., 2014; Yoon et al., 2004).

4.4. Anatomical assessments correlate with functional recovery

Traditionally, spared white matter is reported to be a reliable histological outcome measure in predicting motor function following thoracic SCI (Basso et al., 1995, 1996a; Kloos et al., 2005) the relation between lesion location, tissue sparing and recovery is complex (Fouad et al., 2021; Schucht et al., 2002). Interestingly, spared tissue area and volume measurements distinguished between all but the most severe SCI groups, and the most sensitive histological outcome in our dataset was the percentage of area occupied by lesion, which showed a strong and significant correlation with BBB scores, ladder test results, acetone test responses, and several NC outcomes. This relationship may however be specific to the dorsal lesion approach, and it may also be confounded by the shrinking of the spinal cord itself over chronic time period.

5. Conclusion

In conclusion, we present a standardized in vivo thoracic SCI platform for the comprehensive evaluation of contusive SCI across lesion severities, time points, and functional and histological outcomes.

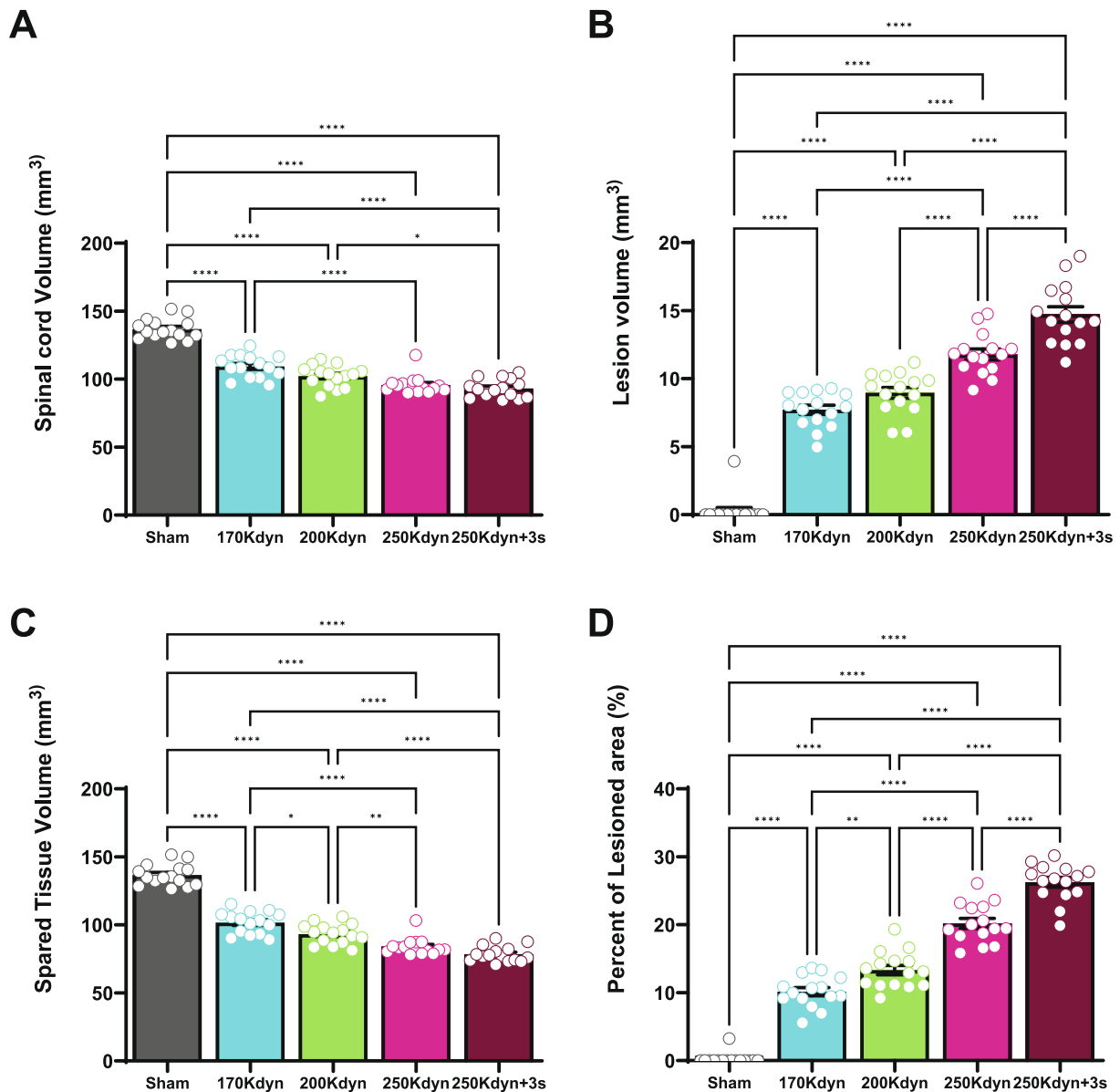


Fig. 10. Graded SCI increases lesion volume and progressively reduces tissue preservation. (A) Total spinal cord volume along the 3 cm segment encompassing the lesion epicenter. All SCI groups show reduced total tissue volume compared to Sham. (B) Lesion volume increases progressively with injury severity. (C) Spared tissue volume decreases in a severity-dependent manner. (D) Percentage of spinal cord occupied by lesion highlights graded differences between SCI groups. Data are presented as mean $\pm$ SEM. $n = 15$ animals/group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. Significant group effects were observed for total spinal cord volume ($F(4, 70) = 82.09, p < 0.0001$), lesion volume ($F(4, 70) = 180.9, p < 0.0001$), spared tissue volume ($F(4, 70) = 156.3, p < 0.0001$), and percentage lesion ($F(4, 70) = 253.1, p < 0.0001$). Significant Tukey's post-hoc comparisons are indicated in the figure; adjusted p -values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Similar platforms in other fields have accelerated research and facilitated independent replication, and this platform may therefore provide a valuable resource for industry as well as investigators who lack specialized in vivo expertise or seek to validate findings in an unbiased manner. However, the complexity of SCI requires careful consideration of treatment mechanisms, targets, and anticipated effect sizes when implementing such a platform, as these factors will strongly influence the likelihood of success, i.e., the detection of treatment benefits.

Without that, a negative result is nearly uninterpretable. A misaligned preclinical model may mask the beneficial effects of otherwise promising interventions.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.expneurol.2026.116025>.

Behavioral, Sensory, and Gait Outcomes Correlate with Lesion Area Following SCI									
		Hindlimb Parameters				Forelimb Parameters			
BBB Score		0.88							
Hindlimb ladder error (%)		0.82				ns			
Von Frey PWT (Week 5)		0.36							
Acetone Frequency (Week 5)		0.61							
Locomotor Activity	Walk Distance	0.28							
	Walk Duration	0.48							
	Walk Speed	0.41							
Body Morphology	Body Area	0.53							
	Body Perimeter	0.49							
	Body Horizontal	0.35							
Paw print count	HL/FL Paw Count ratio	0.89							
	Paw Print count	0.74				ns			
Interlimb coordination	Total Coordinated Steps	ns				0.74			
	Alternate Step Sequence	ns				0.65			
	Cruciate Step Sequence	0.13				0.77			
	Rotatory Step Sequence	0.40				ns			
Paw morphology and imaging	Paw Position Relative Body Center	0.22				0.68			
	Base Width	0.47				0.61			
	Paw Print area	0.34				ns			
	Paw Print Min Diameter	0.38				0.16			
	Paw Print Max Diameter	0.25				0.29			
	Paw Print Intensity	0.36				ns			
Gait geometry and dynamics	Stride Length	ns	0.21	ns	0.13	0.82	0.46	0.64	0.77
	Splay Length	0.15	0.20	0.12	ns	0.75	0.20	0.51	0.73
	Stride Duration	0.08	ns	ns	ns	ns	0.19	0.38	0.33
	Stand Duration	0.23	0.27	0.44	0.40	0.10	ns	0.06	ns
	Swing Duration	0.43	0.35	0.34	0.32	0.27	0.24	0.40	0.43
	Duty Cycle	0.49				0.36			
		All Speed	Low Speed	Medium Speed	High Speed	All Speed	Low Speed	Medium Speed	High Speed

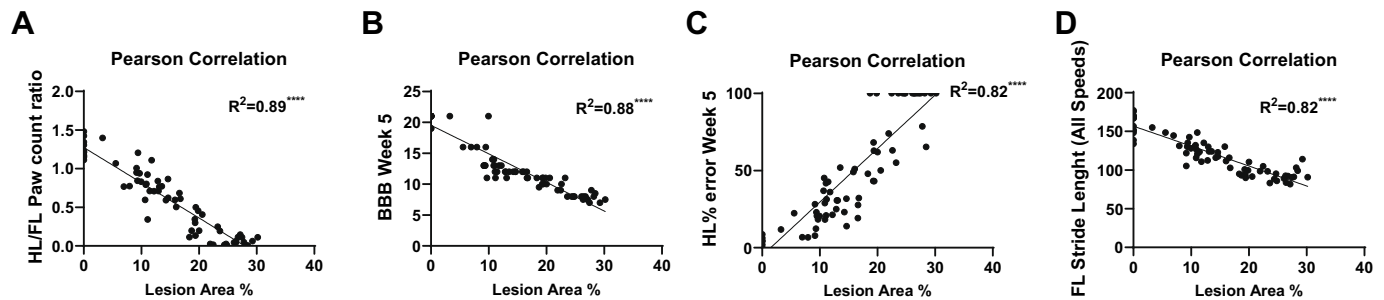


Fig. 11. Locomotor and gait impairments strongly correlate with lesion severity. R^2 coefficients obtained from Pearson correlation analyses between lesion area percentage and behavioral, sensory and NeuroCube gait parameters. Only statistically significant correlations ($p < 0.05$) are listed; non-significant correlations are indicated as “ns”. Color intensity reflects the strength of the association, with darker blue shades indicating higher R^2 values and lighter blue shades indicating lower R^2 values. Panels A–D highlight representative parameters exhibiting the strongest correlations with lesion area: (A) Hindlimb (HL) /Forelimb (FL) paw count ratio, (B) BBB score at Week 5, (C) HL ladder error rate, and (D) mean FL stride length. Pearson correlations shown in panels A–D were significant at **** $p < 0.0001$. The same datasets and sample sizes described for the corresponding behavioral and histological outcomes were used for the correlation analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CRedit authorship contribution statement

Johana Bastidas: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Linda Jones:** Writing – review & editing, Project administration, Conceptualization. **Marco Baptista:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Jason J. Wheeler:** Writing – review & editing, Formal analysis, Data curation. **Rachel Barrett:** Methodology, Investigation, Data curation. **Smriti Mongia:** Methodology, Investigation, Formal analysis, Data curation. **Daniel Havas:** Formal analysis, Data curation. **Karim Fouad:** Writing – review & editing, Conceptualization. **Wolfram Tetzlaff:** Writing – review & editing, Conceptualization. **Taleen Hanania:** Writing – review & editing, Funding acquisition,

Conceptualization. **Megan Ryan Detloff:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Ethics declaration

This study was conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of PsychoGenics, Inc.

Declaration of competing interest

Johana Bastidas, Rachel Barrett, Smriti Mongia, Daniel Havas, and Taleen Hanania are employed by PsychoGenics, Inc. Linda Jones is a

consultant for the Christopher & Dana Reeve Foundation. The authors Jason J. Wheeler, Karim Fouad, Wolfram Tetzlaff, Marco Baptista, and Megan R. Detloff 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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Data availability

Study data were deposited and are currently under review at the Open Data Commons for Spinal Cord Injury (ODC-SCI; RRID: [SCR_016673](#)). By the time of publication, the complete dataset and methods will be publicly available. The ODC-SCI is a secure, cloud-based repository platform designed to share research data (Torres-Espin et al., 2022).

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