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Development of a Rat Forelimb Unloading Model to Understand Mechanical Influences on Postnatal Shoulder Development

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Abstract

Mechanical loading is critically important for skeletal development. Joint unloading, characteristic of many diseases and injuries, creates a unique mechanical environment that leads to altered joint development. While other murine unloading paradigms exist, there is a need for a forelimb unloading model to elucidate the effects of unloading on glenohumeral joint development. We have developed the first forelimb unloading system aimed at isolating these unloading contributions. Specialized rat harnesses attached to a custom, 3D-printed I-beam and two-wheel track system allow for the reduction of weight bearing in the forelimbs without restriction of linear and lateral freedom of movement. The purpose of this study was to validate our custom unloading system via implementation with three-week-old Sprague Dawley rats for a six-week period of constant unloading. Body mass and grooming habits were recorded to assess the rats' well-being. These measures suggested that, after a three-day period of adjustment, the unloading system had minimal impact on normal activity. Body mass measurements for unloaded rats were generally within the measurement range for the control rats, indicating that the system does not impact normal food consumption and growth. Bone structure in the unloaded glenohumeral joints will be characterized from micro-computed tomography scans. Preliminary data indicates altered macrostructure, including geometry of the humeral head and glenoid region of the scapula following unloading. These results will aid in characterizing the changes in bone development that occur due to glenohumeral joint unloading. Ultimately, results from this study will be used to determine the independent contributions of unloading to bone deformities following brachial plexus birth injury.

Keywords: forelimb unloading, bone, joint development

1. Introduction

1.1. Effects Of Mechanical Loading On Skeletal Development

According to Wolff's Law, bone adapts in response to the mechanical loads it experiences[1], leading to more bone deposition in regions with increased tissue stresses and strains and more bone resorption in regions with reduced stresses and strains[2]. These opposing mechanisms are especially important during skeletal development when bone growth rates are highest. In normal loading environments created by load-bearing activity and muscle-generated movements, healthy bones and joints will develop properly. With altered loading environments, such as in limb disuse and muscle paralysis, skeletal development is altered, resulting in abnormal bone and joint morphology and function. Abnormal bone growth resulting from changes in the bone's mechanical environment manifests both as deformities in gross morphology and deterioration in underlying microstructure. Abnormal bone morphology and reduced bone formation are observed in the scapula and humerus in a "muscleless" murine model that lacks forces from muscle contraction[3]. Morphological deformities also occur in the bones of the human acetabulofemoral joint due to irregular loading caused by cerebral palsy[4] or unnatural limb positioning in the womb[5]. Reduced mechanical loads experienced

by humans on bedrest stimulate changes in bone microstructure, including increased trabecular number and decreased trabecular thickness[6]. Certain musculoskeletal diseases and injuries, such as peripheral nerve injuries, can induce altered loading conditions that are often only one part of a complex cascade of changes that occur. Isolating the specific impacts of unloading on bone and joint development, separate from muscle or nerve effects that may occur, is a key first step for developing more targeted, effective treatments of complex musculoskeletal injuries.

1.2 Current Unloading Paradigms

The isolated effects of unloading on bone have been studied using several different animal models, ranging from both partial unloading of all limbs[7] to complete unloading of the hindlimbs[8]. To date, no study has used a complete forelimb unloading model, which would be relevant for cases of disuse or injury affecting bones and joints in the arm. One previous study presented a mouse model for bipedal locomotion on the hindlimbs that effectively unloaded the forelimbs[9], although the long-term effects of forelimb unloading on the skeleton were not examined. This model is not appropriate for studying forelimb unloading, because it disrupts the natural posture of the animal and is not intended for extended periods of use. A new forelimb unloading model is needed to characterize the effects of altered mechanical loading on bone and joint development in the arm. The glenohumeral joint in the shoulder is of particular interest, because brachial plexus birth injury (BPBI), the most common nerve injury in children[10], is known to cause glenohumeral deformities[11,12] that impair joint range of motion and arm function[11,12,13]. Rat models are ideal for studying the glenohumeral joint, because rat shoulders are anatomically similar to human shoulders with homologous skeletal and muscular features[14, 15], and rats reach musculoskeletal maturity rapidly, progressing the equivalent of 1 human year in only 10.5 days[16]. Using a rat model for unloading can provide insight regarding the *in vivo* joint changes that may be expected in humans under similar conditions.

1.4 Forelimb Unloading Contributions To BPBI

Previous work in an established rat model of BPBI showed that morphological alterations in the scapula and humerus include a flatter, abnormally-shaped glenoid and a subluxated, smaller humeral head, leading to joint misalignment and partial dislocation characteristic of clinical BPBI cases[17]. These deformities likely result from a combined influence of 1) mechanical unloading from disuse of the limb, 2) abnormal muscle growth and loading, and 3) denervation in affected bones and muscles, which are all known to impact bone development. The isolated effects of these different factors remain unknown and cannot currently be assessed in either clinical or animal studies of BPBI. Application of a new murine forelimb unloading model in the context of a larger BPBI study will help distinguish the impact of forelimb unloading, separate from other factors, on glenohumeral development and function.

The purpose of this study was to design, implement, and validate a custom forelimb unloading model in rats. The overall goal is to use this model to determine the effects of mechanical unloading on glenohumeral development, both macrostructurally and microstructurally, and ultimately to use these results in a larger study to help isolate the unloading contributions to BPBI-related shoulder dysfunction. This work will significantly advance our understanding of the role of mechanical loading in the development of shoulder deformities and will inform treatments that may mitigate loss of arm function with these types of musculoskeletal injuries.

2. Methods

Following multiple design iterations and pilot trials, the proposed model was selected to optimize effective unloading and the well-being of the rats. Two key components comprise the final model: specialized harnesses and a unique suspension system. Each component has been selected and assembled with careful concern for the unique requirements with using a murine model. Following construction, the design was implemented in a validation study to assess its efficacy as a tool for understanding the role of mechanical loading in the postnatal development of the glenohumeral joint.

2.1 Harness

The purpose of the harness was to facilitate unloading by attaching it to the suspension system yet conserve comfort and mobility of the rats during unloading. When choosing a suitable harness, several important aspects were considered. Since the rats wore the harnesses continuously throughout the study, the harnesses had to allow free access

to food and water without inhibiting grooming and normal cage activity. Custom and commercial harnesses were considered, but existing commercial harnesses were chosen for their availability, low cost, vast array of sizes, and similarity to custom designs without the need for an extended amount of manufacture time and specialized equipment.

Commercial rat harnesses (LOMIR Biomedical Inc., Notre-Dame-de-l'Île-Perrot, Quebec, Canada) were used to attach the suspension system to the rats. Harnesses were manufactured with Lycra®, a type of durable and breathable spandex. The harnesses were customized by the manufacturer, featuring sleeves to reduce chafing under the arms and longer torso coverage to distribute the unloaded weight. Three hook-and-eye fasteners and supplemental hooks were placed at equal increments along the back, enabling secure closure and a two-point tether system. Metal wire was wrapped around the supplemental hooks in a corseted fashion to secure the harness on the rat.

The rats wore harnesses starting at three weeks of age, following weaning. To accommodate the rapid growth of the neonates, with increasing body size and weight from three to nine weeks of age, several harnesses sizes were used. The appropriate size was selected based on fit and comfort. At three weeks of age, an XX-Small harness was appropriate (Figure 1A). At six weeks of age, harness size was increased to an X-Small, which was an appropriate fit for the remainder of the study for the female rats (Figure 1B). Harnesses for male rats were changed to a size Small at seven weeks, and this size was used for the remaining two weeks. The harness system was monitored to ensure the rats were fully mobile and able to perform normal daily activities without pain or discomfort.

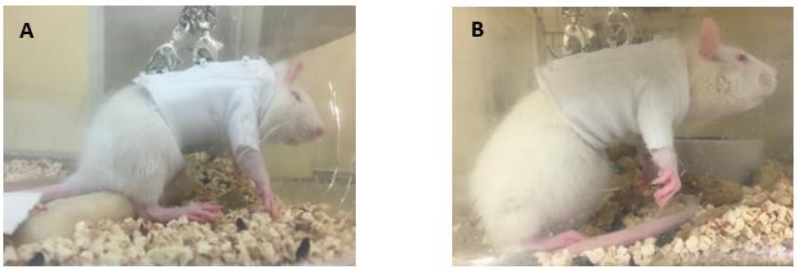

Figure 1. Multiple harness sizes for growing rats

Figure 1. The growing rat is secured in the suspension system using different sized harnesses, shown here on a female rat at (**A**) 4 weeks of age in size XX-Small and (**B**) 8 weeks of age in size X-Small.

2.2 Custom Suspension System

The custom suspension system was designed to prevent weight bearing in the forelimbs. The design process focused on using lightweight materials and minimizing friction to enhance cage mobility. Initial designs for the suspension system were inspired by aspects of unloading systems from past studies, including linear movement along the length of the cage, additional transverse movement provided by a spring, and adjustability using a tensioning cable[7]. The bipedal nature of a previous spinal cord injury support system was also integrated into the design to allow for complete forelimb unloading[9]. A primary goal of the new design was to allow more natural movement of the rats throughout the cage, preserving a more horizontal posture while ensuring complete unloading of the forelimbs. The final design permitted the rats to travel linearly lengthwise in the cage, via a custom I-beam track system, and side-to-side in the cage, via an adjustable chain. An approximately normal posture was maintained during unloading to optimize comfort and enable normal movement as much as possible.

The first few iterations of the suspension system featured commercially available products, including drawer slides, door tracks, and turnbuckles. However, these parts were large and heavy and inhibited the movement of young rats throughout the cage. In the final iteration, scaled-down parts made of lightweight materials were chosen. Because the rats weighed a maximum of 250 grams, the system was still sufficiently strong to withstand movement forces imposed by the animals. The result was a unique system consisting of a combination of purchased and 3D-printed plastic parts that allowed the rats to be fully mobile during forelimb unloading. A computer model was generated to help visualize the design and facilitate 3D printing of the various components (SOLIDWORKS® 3D, Dassault Systèmes SOLIDWORKS Corp., Waltham, MA).

The I-beam track system was designed to be easily replicable for use in multiple cages and smooth to minimize friction during movement (Figure 2). It ran lengthwise through the center of the cage, serving as a track system. Sixteen circular extruded hooks were added to the top of the I-beam to keep the suspension system out of reach of mature rats, and 3/8" diameter wooden dowels were placed through the extruded holes to secure the I-beam into the lid of the cage. To facilitate movement along the length of the cage, a carriage consisting of two wheels joined via

custom wheel attachment parts, aluminum rods, and custom laser-cut acrylic nuts was fitted inside the I-beam track. The track was sized as small as possible while still accommodating the commercially available wheels. The wheel attachment part was designed to connect the wheels on each side of the I-beam, maintaining stability in the system.

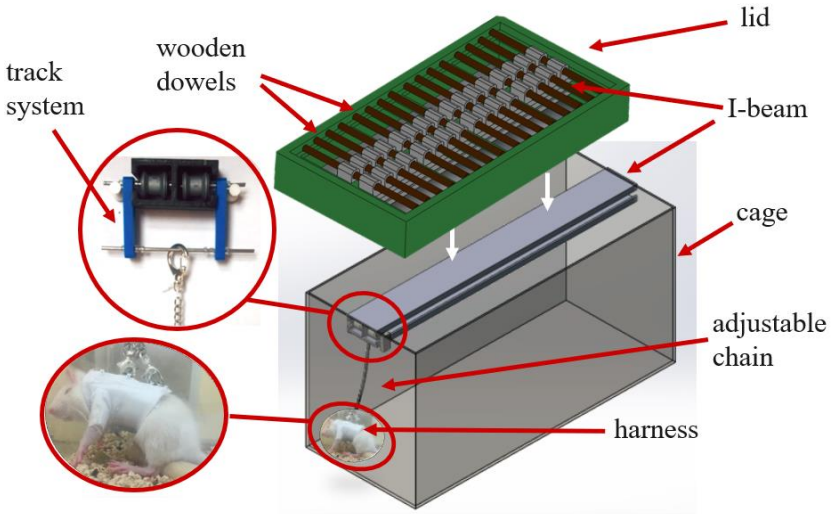

Figure 2. Suspension system apparatus

Figure 2. The novel forelimb unloading system features a custom I-beam and track system that fits into a standard cage lid; an adjustable chain to maintain unloading as the rats grow; swivel hooks to enable full movement throughout the cage and easy removal from the cage; and custom rat harnesses offering security and support with minimal impact on daily activity.

Each rat harness was connected to the custom suspension system using two lightweight rhodium-plated iron curb chains. The chain was selected to provide an adjustment mechanism to accommodate rat growth, since the chain lengths could be set for the desired unloading posture for each rat individually. Both chains connected to the suspension system using a metal swivel hook that was hooked to the rod beneath the wheel attachment piece. The swivel hook was chosen to allow for effortless removal of the rat from the cage to perform various procedures and biomedical assessments throughout the study, such as body mass measurements. At the bottom end were two chains that created a two-point tether system in which each diverging chain attached to the rat harness via metal hooks that looped through the metal wire "corset". Two chains were used to distribute the rat's body weight along the lower body while maintaining a relatively normal posture. This prevented the weight from being concentrated at the rat's chest, which would cause discomfort and more chafing of the skin beneath the harness.

The custom I-beam and wheel attachment parts were 3D-printed, and the remaining supplies were purchased. The I-beam and wheel attachment parts were made of polylactic acid plastic to minimize the weight of the system, thereby minimizing resistance to movement.

2.3 Validation Study

All procedures were approved by the Institutional Animal Care and Use Committee at North Carolina State University. Six Sprague Dawley rats (2 female, 4 male) from three litters were exposed to forelimb unloading beginning immediately after weaning at three weeks of age. The rats were anesthetized with inhaled isoflurane and fitted to appropriately sized harnesses. Each rat was singly housed in a custom cage, where the harness was connected to the custom suspension system. The rats were exposed to a 12-hour light/12-hour dark cycle and administered rat chow and HydroGel® (ClearH2O®, Inc., Westbrook, ME) *ad libitum*. Forelimb unloading was maintained continuously for the duration of the study.

Rats were removed from the suspension system every other day for body mass measurements and immediately returned to their unloading environment to minimize weight bearing. Pain signs were monitored every other day. Evidence of porphyrin secretion from the eyes or nose, a common indicator of stress[18], was recorded. Harnesses were removed weekly, and the fur and skin beneath the harness were inspected, and lesions on the skin were promptly

treated with topical triple antibiotic ointment. Harness size and chain length were adjusted as needed to maintain unloading as the rats grew. To monitor cage activity and grooming habits while the rats were alone, a video camera (GoPro® Hero4 Black Edition, San Mateo, CA) was placed in front of two randomly selected cages for a 2-hour recording duration at two random timepoints in the study: once immediately following a weekly harness check and once in between harness checks. At nine weeks of age, after six weeks of unloading, the rats were euthanized with CO_2 inhalation. Left and right scapulae and humeri were harvested and fixed in 10% neutral buffered formalin for two days, followed by storage in 70% ethanol at 4°C until analysis. Right scapulae and humeri were assessed with quantitative micro-computed tomography (micro-CT). Bones were scanned at 45 kVp and 177 µA using a 0.5-mm Al filter and 800-ms integration time (µCT 80, SCANCO Medical AG, Brüttisellen, Switzerland). The scans were reconstructed using an 18-µm voxel size, reoriented anatomically, and analyzed in Mimics (Materialise, Leuven, Belgium) for the following macrostructural metrics: humeral head thickness and width, glenoid radius of curvature, and glenoid inclination angle[17,19] (Figure 3).

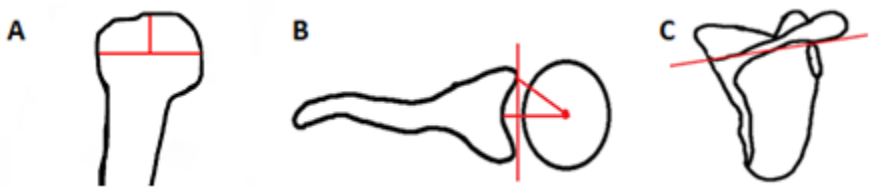

Figure 3. Bone measurements of interest

Figure 3. Micro-CT scans were used to quantify bone macrostructural measurements, including (**A**) humeral head thickness (vertical) and width (horizontal), (**B**) glenoid radius of curvature, and (**C**) glenoid inclination angle.

Body mass measurements were compared between the unloading group and a group of 6 littermates who served as control rats (4 female, 2 male). Control rats were group housed (3 per cage) in the same facility and with the same light/dark cycle as the unloading rats. They had free access to food and a standard water bottle rather than HydroGel® like the unloading group. Control rats were euthanized with CO_2 inhalation at 8 weeks of age, when they reached skeletal maturity. The length of the study for unloading rats was one week longer to achieve six weeks of unloading.

3. Results

Throughout the six-week unloading period, the custom forelimb unloading apparatus enabled the rats to maintain freedom of movement within the cage with minimal disruptions to normal activity. During the first week, four rats (2 female, 2 male) escaped due to the combination of low body weight and laxity in the harnesses created after placing them in the suspension system. As a result, these rats were temporarily exposed to limb loading while they were out of the suspension system. As the rats grew during the study, the fit of the harnesses improved, and this problem did not persist. In the third week of unloading, the four male rats were removed from the unloading environment for four days due to chafing of the harness sleeves against the skin at the elbow joint and excessive porphyrin secretions around the eyes and nose. After the sleeve lengths were cut to avoid further chafing, the harnesses were refitted on the rats and remained on during the four-day healing period until unloading was resumed. Other than these brief instances, unloading was maintained for the six-week duration.

The forelimb unloading system did not substantially alter normal growth patterns in either female or male rats. Body mass measurements followed an approximately linear trend, similar to those observed in the control rats (Figure 4). Except for three measurements, body mass for the female rats was always within one standard deviation of the control mean (Figure 4A). Similarly, male body mass for the unloaded rats generally fell within the range predicted by the control group (Figure 4B). However, body mass for one male rat trended downward in the final three days of the study.

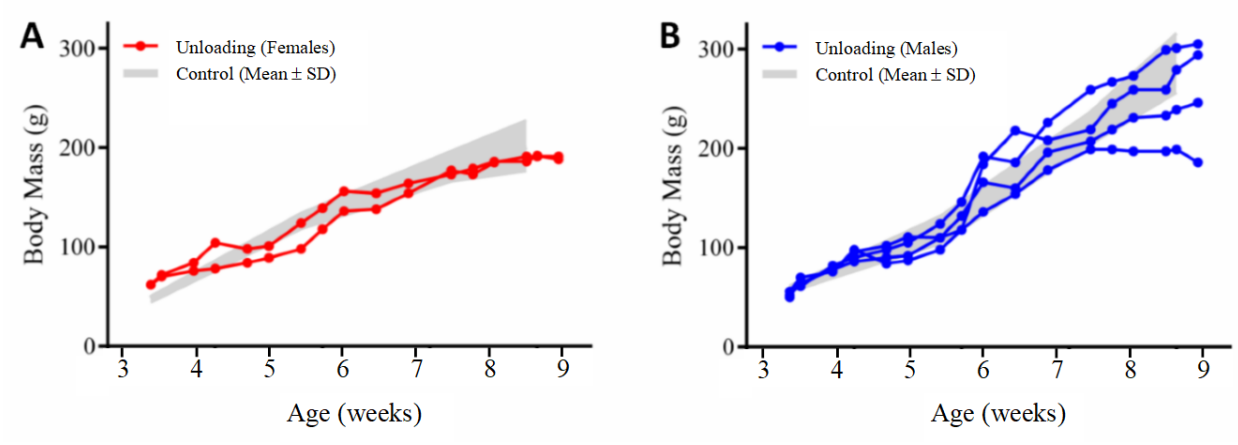

Figure 4. Rat growth for female and male rats in the unloading system

Figure 4. Body mass of the (**A**) two female rats and (**B**) four male rats during 6 weeks of unloading followed the characteristic increasing trend observed in the sex-matched control rats, with most measurements falling within one standard deviation of the control mean (shaded). Body mass could not be compared to the control groups during the final week of unloading due to the difference in study duration.

The unloading system appeared to be well tolerated by the rats throughout the study. The rats maintained regular grooming habits, and the exposed fur and skin appeared healthy with no lesions or fur loss. Fur beneath the harness was matted, but the skin was generally unharmed, other than the temporary skin irritation mentioned above. The first harness change was followed by evidence of porphyrin discharge around the nose or eyes in all rats, but this stopped after three days. Porphyrin discharge lasting two days was observed in one of the male rats following the second harness change. These levels of increased and continuous discharge indicated that the rats were experiencing stress, likely related to initial discomfort with the new environment.

Cage activity followed a similar pattern after each weekly check: after a brief adjustment period following introduction to the unloading environment, the rats became more active. This became especially clear upon review of the time-lapsed GoPro® video footage taken immediately after a weekly harness check. After a brief 10-minute period while the rats were still recovering from anesthesia, they were mobile in their cages. In both sets of videos, the rats demonstrated normal cage activity, with alternated periods of rest and frequent periods of activity, while still eating and drinking normally.

Measurements of bone macrostructure from micro-CT scans are underway. Compared to the group of control rats (n=6), forelimb unloading rats (n=1 analyzed thus far) had altered morphology in the humerus and scapula (Table 1). In the humerus, compared to the control rats, the unloaded rat had a 22.3% smaller humeral head thickness and 23.3% smaller humeral head width. In the scapula, the unloaded rat had a 53.8% greater glenoid radius of curvature. The glenoid inclination angle was slightly higher (more inclined) for the unloaded rat compared to the control rats, but the angles were similar with only a 3.6% difference.

Table 1. Bone morphology metrics for the unloading rat (n=1) were different than those for control rats (n=6).

	Humeral Head Thickness (mm)	Humeral Head Width (mm)	Glenoid Radius of Curvature (mm)	Glenoid Inclination Angle (°)
Control	4.59	4.52	3.12	-32.78
Forelimb Unloading	3.57	3.47	4.80	-31.6
Percent Difference	-22.3%	-23.3%	+53.8%	+3.6%

4. Discussion and Conclusion

The design of this novel forelimb unloading model was necessary for use in discerning the effects of glenohumeral joint unloading on postnatal bone development. Extensive research, design, and testing preceded construction and validation of the finalized, custom suspension system. Since the effective design can be manufactured by students in less than two days for a cost of less than fifty dollars per cage, it is a feasible method for unloading studies in many contexts.

This study validated the efficacy of the final design. Indicators of animal well-being suggest that the suspension process is initially stressful for the rats, but then they become acclimated after only three days in the system. This acclimation period is on par with acclimation durations used in other loading and unloading studies. After this adjustment period, the rats moved freely throughout the cage while grooming and eating normally. Body mass measurements confirmed that this model had minimal impact on food consumption and growth in all but one rat. This rat experienced a 7% decrease in body mass toward the end of the study, which may indicate excessive stress or discomfort. Since this was only observed in one rat, it is not likely that unloading was solely responsible for the decrease.

Preliminary micro-CT results suggest that forelimb unloading affects bone morphology in regions of the scapula and humerus that facilitate movement of the glenohumeral joint, consistent with expected bone changes associated with unloading. Future work will focus on further bone analysis for the rats in this study. Bone morphology, including humeral head thickness and width, glenoid radius of curvature, and glenoid inclination angle, as well as microstructure, including trabecular number, thickness, and separation, will be characterized for all six rats using micro-CT. The effects of unloading on bone morphology and microstructure will be assessed by comparing these results to those for control rats. Histological muscle analysis will also be conducted to investigate the effects of unloading on muscle development. Optimization of the unloading system will continue, as well. Since the results indicate that the model is better suited for female rats, which showed no evidence of skin irritation and thus did not experience a loading period to allow for skin healing, subsequent updates to the model will seek to adapt the system for male rats by switching to neoprene harnesses to reduce chafing. Continued experimentation will use a larger sample size and an improved control group, consisting of singly housed rats with free access to HydroGel® instead of water. The improved control will also be euthanized at the same age as the unloading group for better assessments of body mass trends beyond eight weeks of age.

Moving forward, the validated system will be used in a larger study to isolate the independent contributions of unloading to BPBI. After the larger unloading experiment is completed, the effects of unloading on shoulder development will be determined using several analyses, including micro-CT and muscle and bone histology. The results will be compared to those from the larger study that includes rats receiving nerve injury mimicking BPBI to separate the effects of forelimb unloading from those of muscle and bone denervation and altered muscle growth. These comparisons will help isolate the contribution of limb unloading to BPBI-induced deformities that impact shoulder movement and function and alter the underlying microstructure and metabolism of bone. Characterizing

these changes may improve understanding of how normal bone and muscle interaction during postnatal development is altered with nerve injury, which is the first step for developing better treatment strategies for children with BPBI. On a broader scale, this model may help distinguish the isolated effects of unloading in numerous diseases and injuries, thereby giving insight for improvements to existing medical care.

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