Journal of Visualized Experiments

A battery of motor tests in a neonatal mouse model of cerebral palsy

--Manuscript Draft--

Manuscript Number:	JoVE53569R2
Full Title:	A battery of motor tests in a neonatal mouse model of cerebral palsy
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	behavior; neonatal mouse; motor deficits; cerebral palsy; hypoxia; ischemia; inflammation; neonatal motor tests
Manuscript Classifications:	3.10: Nervous System Diseases; 3.10.228.140.140.254: Cerebral Palsy; 3.10.228.140.624.500: Hypoxia-Ischemia, Brain; 3.10.597.606: Neurobehavioral Manifestations; 95.51.4: animal models
Corresponding Author:	Tanya S. Ferguson, Ph.D. Shriners Hospitals Pediatric Research Center Philadelphia, PA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	tanyaf@temple.edu
Corresponding Author's Institution:	Shriners Hospitals Pediatric Research Center
Corresponding Author's Secondary Institution:	
First Author:	Tanya S. Ferguson, Ph.D.
First Author Secondary Information:	
Other Authors:	Danielle N Feather-Schussler
Order of Authors Secondary Information:	
Abstract:	As the sheer number of transgenic mice strains grow and rodent models of pediatric disease increase, there is an expanding need for a comprehensive, standardized battery of neonatal mouse motor tests. These tests can validate injury or disease models, determine treatment efficacy and/or assess motor behaviors in new transgenic strains. This paper presents a series of neonatal motor tests to evaluate general motor function, including ambulation, hindlimb foot angle, surface righting, negative geotaxis, front- and hindlimb suspension, grasping reflex, four limb grip strength and cliff aversion. Mice between the ages of post-natal day 2 to 14 can be used. In addition, these tests can be used for a wide range of neurological and neuromuscular pathologies, including cerebral palsy, hypoxic-ischemic encephalopathy, traumatic brain injury, spinal cord injury, neurodegenerative diseases, and neuromuscular disorders. These tests can also be used to determine the effects of pharmacological agents, as well as other types of therapeutic interventions. In this paper, motor deficits were evaluated in a novel neonatal mouse model of cerebral palsy that combines hypoxia, ischemia and inflammation. Forty-eight hours after injury, five tests out of the nine showed significant motor deficits: ambulation, hindlimb angle, hindlimb suspension, four limb grip strength, and grasping reflex. These tests revealed weakness in the hindlimbs, as well as fine motor skills such as grasping, which are similar to the motor deficits seen in human cerebral palsy patients.
Author Comments:	Please find attached our revised manuscript. Also, in the Author section, there is no "order" section. However, Danielle Feather should be the first author and Tanya Ferguson should be the corresponding author. I cannot correct it. Please find attached our second revision of our manuscript, the first revision that includes reviewer comments. All changes have been noted within the manuscript, as indicated by a text color change (red). We made revisions recommended by the reviewers. However, with Reviewer #1, we had some difficulty addressing the comments due to either confusing or incomplete statements by the reviewer, as well as

	citing text line numbers that did not match our version of the paper. We did the best we could to interpret the comments, but in some places could not. These places are noted within our rebuttal document.
Additional Information:	
Question	**Response**
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	07-24-2015
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

Shriners Hospitals for Children®

School of Medicine TEMPLE UNIVERSITY®

April 10, 2014

Dear JoVE Editorial Board:

Please accept for review our manuscript entitled “A battery of motor tests in a neonatal mouse model of cerebral palsy”. I was contacted by Ola Jachtorowicz, Deputy Director of Editorial – Life Sciences prior to the Society for Neuroscience in Washington DC this past November. We met at the meeting where she asked us to consider submitting this manuscript to JoVE. She has remained in touch with us while we prepared and finalized the manuscript.

This manuscript represents a quantitative battery of motor tests for neonatal mice that can be used to evaluate mouse models of pediatric neurological and neuromuscular disorders, as well as other pediatric diseases. There is a distinct lack of standardized motor tests with which to assess motor behavior in pediatric models of disease, thus making cross-talk between investigators difficult. By combining neonatal motor tests into one document, with a number scoring system to help determine severity and progressiveness of a disease, as well as to determine improvement after treatment, it will allow quantitative documentation of neonatal motor diseases and enable researchers to better communicate results within a single model. In addition, the included scoring sheet will allow baseline deficits to be noted, as well as any changes following treatment or over time.

One of the problems with behavior tests is that the subtle nuances of how to perform a particular test do not translate well with just words or static pictures. By having our manuscript with representative results using our mouse model of cerebral palsy accepted by JoVE, we can demonstrate proper techniques using live neonatal mice and video recording. It will enable researchers to accurately perform each test and strengthen the results so that they can be compared between research groups.

Thank you for your consideration of our submission,

Tanya S. Ferguson, Ph.D.

Assistant Professor
Shriners Hospitals Pediatric Research Center
Temple University School of Medicine
3500 N. Broad Street, 6th Floor
Philadelphia, PA 19140
Work: 215-926-9383 Cell: 610-405-8674
Work: tanyaf@temple.edu Personal: phillyfergtanya@gmail.com

Suggested Reviewers:

1. Peter Kiss, Department of Anatomy, University of Pecs, Szigeti u 12, 7624 Pecs, Hungary. Email: peter.kiss@aok.pte.hu
2. Guillaume Sebire, Department of Pediatrics and Department of Neurology and Neurosurgery, McGill University, Montreal, Canada. Email: Guillaume.sebire@usherbrooke.ca
3. Wenbin Deng, Associate Professor, 653B Shriner's Hospital, Sacramento Campus, UC Davis, Sacramento, California, USA. Email: wbdeng@ucdavis.edu.
4. Zhengwei Cai, Professor, University of Mississippi Medical Center, Mississippi, USA. Email: zcai@ped.umsmed.edu.
5. Marc Jamon, Faculté de Médecine de la Timone, Institut National de la Santé et de la Recherche Médicale U1106, Aix-Marseille University, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France. Email: Marc.jamon@univ-amu.fr.
6. Maria-Paz Viveros Departamento de Fisiología (Fisiología Animal II), Facultad de Ciencias Biológicas, Universidad Complutense, Ciudad Universitaria, C/Jose Antonio Novais 2, 28040 Madrid, Spain. Email: pazviver@bio.ucm.es.

TITLE:
A battery of motor tests in a neonatal mouse model of cerebral palsy

AUTHORS:
Feather-Schussler, Danielle N.
Biochemistry Department, Shriners Hospital Pediatric Research Center
Temple University School of Medicine
Philadelphia, PA
dfeather@temple.edu

Ferguson, Tanya S.
Shriners Hospital Pediatric Research Center
Temple University School of Medicine
Philadelphia, PA
tanyaf@temple.edu

CORRESPONDING AUTHOR:
Ferguson, Tanya S.
Shriners Hospital Pediatric Research Center
Temple University School of Medicine
Philadelphia, PA
215-926-9383
tanyaf@temple.edu

KEY WORDS:
Behavior; neonatal mouse; motor deficits; cerebral palsy; hypoxia; ischemia; inflammation; neonatal motor tests

SHORT ABSTRACT:
Presented is a concise battery of mouse neonatal motor tests. Using these tests, neonatal motor deficits can be demonstrated in a variety of neonatal motor disorders. By having a standardized set of tests, results from different studies can be compared, allowing for better and accurate reporting between groups.

LONG ABSTRACT:
As the sheer number of transgenic mice strains grow and rodent models of pediatric disease increase, there is an expanding need for a comprehensive, standardized battery of neonatal mouse motor tests. These tests can validate injury or disease models, determine treatment efficacy and/or assess motor behaviors in new transgenic strains. This paper presents a series of neonatal motor tests to evaluate general motor function, including ambulation, hindlimb foot angle, surface righting, negative geotaxis, front- and hindlimb suspension, grasping reflex, four limb grip strength and cliff aversion. Mice between the ages of post-natal day 2 to 14 can be used. In addition, these tests can be used for a wide range of neurological and neuromuscular pathologies, including cerebral palsy, hypoxic-ischemic encephalopathy, traumatic brain injury,

spinal cord injury, neurodegenerative diseases, and neuromuscular disorders. These tests can also be used to determine the effects of pharmacological agents, as well as other types of therapeutic interventions. In this paper, motor deficits were evaluated in a novel neonatal mouse model of cerebral palsy that combines hypoxia, ischemia and inflammation. Forty-eight hours after injury, five tests out of the nine showed significant motor deficits: ambulation, hindlimb angle, hindlimb suspension, four limb grip strength, and grasping reflex. These tests revealed weakness in the hindlimbs, as well as fine motor skills such as grasping, which are similar to the motor deficits seen in human cerebral palsy patients.

INTRODUCTION:

Developing new models of pediatric injury or disease using rodents is often difficult due to the amazing ability of both rats and mice to rapidly recover from neurological injury. Therefore, in order to validate any new pediatric disease model, thoroughly examining the cellular and molecular changes must go hand-in-hand with behavioral outcomes. In many ways, functional behavioral recovery may be more important than underlying cellular changes in terms of therapeutic or translational relevance. As researchers learn more about injury in the adult and neonate, it is clear that their responses are very different and cannot be extrapolated between the two. For example, neonatal mice display different levels of nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and glial cell line-derived neurotrophic factor following spinal cord injury [1,2]. Additionally, neonates have significant blood-brain barrier leakage after stroke [3], demonstrate cortical neuron rearrangement after peripheral nerve injury [4], and have a delayed or slowed astrogliosis following spinal cord injury and hypoxia-ischemia [5,6]. Therefore, it is important that translational pediatric research use developmentally equivalent models and that those models are evaluated for both cellular/molecular changes *and* age-appropriate behavioral tests.

Cerebral Palsy (CP) is a motor disorder that affects 3:1000 live births annually (NIH). Children with CP exhibit a range of symptoms and co-morbid conditions, depending on the severity of the disease. Difficulty with movement and coordination are the most common signs, along with delays in reaching motor developmental milestones. Other signs include abnormal muscle tone (either increased or decreased), reduced fine motor skills, difficulty walking, excessive drooling and swallowing, and speech delays (NIH). The underlying cause of CP is believed to be a lack of oxygen and/or blood flow to the brain during the pre- or peripartum period, or up to one-year post-partum. In addition, inflammation is now believed to be a key component in the development of CP.

The majority of CP cases are associated with white matter damage around the ventricles, known as periventricular leukomalacia (PVL). This neurological hallmark suggests that the initial insult leading to CP occurs during the period of brain development when the oligodendrocytes are most vulnerable to insult. The period of rapid oligodendrocyte growth in a human, also the period when oligodendrocytes are the most susceptible to injury, is between 24-32 weeks gestation. In the rodent, the equivalent period is post-natal days 2-7 [7], and is when CP is induced in this model.

The neonatal mouse model of CP that was used to conduct the tests outlined here combines hypoxia and ischemia with inflammation to create an injury that better mimics the neurodegeneration seen in human CP. This model addresses some of the major shortcomings observed in other animal models of CP, which lack distinct motor deficits that resembles human CP patients, as well as distinct white matter damage. Previous studies by a collaborator using the same model have demonstrated that the addition of inflammation enhances white matter damage, thus better emulating the PVL seen in children with CP [8]. Building on the previous data, this paper presents a comprehensive battery of neonatal motor tests in order to evaluate changes in motor behavior as the animal ages.

PROTOCOL:

NOTE: All animal surgeries were performed in accordance with Temple University's ULAR department and IACUC policies and procedures. C57BL/6 dams and sires were purchased from Charles River Laboratories and were housed in breeding cages with a 12 hr light/dark cycle (light on 7:00-19:00) with free access to food and water. Breeding pairs produced litter sizes between 5-10 pups.

1. Cerebral Palsy Induction Surgery

NOTE: Cerebral palsy was induced using post-natal day (PND) 6 mouse pups, as previously described[8,9] (http://www.jove.com/video/1951/mouse-models-of-periventricular-leukomalacia).

1.1) Place a pup in a glass bowl on ice with a laboratory wipe to protect the pup's skin. Check for appropriate anesthetic plane by foot pinch and lack of movement. Move the pup to a padded ice pack for surgery.

1.2) Sterilize the skin of the pup using 70% ethanol. When dry, use a #11 sterile surgical blade and make a 1cm incision in the neck.

1.3) Using a stereoscopic surgical microscope, isolate the right common carotid artery with a small hook and cauterize using a portable hand-held cauterizer. Visually confirm that the artery is occluded. Sham surgery includes visualization and isolation of the common carotid artery without cauterization.

1.4) Realign the skin and close using suture glue (n-butyl cyanoacrylate).

1.5) Place the pup on a 34 ^{o}C heating pad for 30 mins to monitor for spontaneous breathing and normal movement.

1.6) Return the pup(s) to dam for 30 mins.

1.7) Place the pups on a heating pad or other warming device set at 34 ^{o}C inside a hypoxia chamber set at 6% oxygen for 35 mins. Oxygen is replaced by nitrogen. Closely monitor the chamber oxygen level and temperature for consistent injury results.

1.8) Remove the pups from the hypoxia chamber and return them to the heating pad.

1.9) Intraperitoneally inject lipopolysaccharide diluted in sterile saline at 1μg/kg and return the pup to the dam. Sham injections are injections of saline only.

2. Neonatal Motor Tests

NOTE: On PND 8, 48 hrs after CP induction, mouse pups are tested for neurobehavioral development. Pups are tested within a four-hour block before noon in order to eliminate time of day differences in behavior. Pups are removed from the dam for no more than 15-mins at a time to prevent rapid loss of body heat and hunger/separation issues. In addition, pups are allowed to rest in between tests so that maximal efforts will be elicited on each test. The basis of the neonatal motor tests is adapted using Fox's battery of tests [10,11] and Wahlsten's adaption of Fox's tests [12], as well as Treat-NMD and other behavior publications (as noted in the text for each test). Fox's battery of tests are appropriate for PND 2-21. Of Fox's tests, the battery present here includes: righting reflex, grasping reflex, negative geotaxis (called vertical screen test in Fox's battery) and four limb grip strength (modified from Fox and Wahlsten's screen climbing tests). Here, ambulation, front-limb strength, and hindlimb strength are also tested to distinguish reflexive motor behavior between sham and CP mouse pups. To eliminate improvements on testing due to learning, tests were limited to a maximum of 3 trials where noted. All other tests had only one trial per animal.

2.1 Ambulation (Figure 1) (adapted from a rat protocol [13]):

NOTE: Crawling is a behavior developed early in the mouse pup between PND 0-5, at which point mice begin to transition to walking, from 5-10 days old [14]. At PND 8, the ambulation test takes advantage of this transitional time course. Ambulation can, however, be scored throughout the lifetime of a mouse and can be determined at any age. As there is no potential for learning, the ambulation test can be repeated as many times as needed through the course of the experiment.

2.1.1) Place mice in a clear enclosure where mice are visible from the top as well as the side (an enclosed plexiglass walkway was made).

2.1.2) Score ambulation for 3 mins using the following scale: 0 = no movement, 1 = crawling with asymmetric limb movement, 2 = slow crawling but symmetric limb movement, and 3 = fast crawling/walking.

NOTE: Here, symmetric limb movement is described where hindpaws meet frontpaws during each step, and each step smoothly transitions to the next step. A mouse displaying asymmetric limb movement has erratic paw placement and transitions from one step to the next are not smooth.

2.2. *Hindlimb foot angle* (Figure 2)**:**

NOTE: There is an apparent developmental change in the hindlimb posture as the mouse matures from crawling to walking, where the hindlimbs are positioned under the body when walking and the angle between the hindlimbs is less than the angle seen in crawling. Even though the hindlimb foot angle changes over time, mouse pups of the same age with different injury or diseases can be compared. Similar to the ambulation test (3.1), there is no potential for learning. Thus, the hindlimb foot angle test can be repeated as many times as needed through the course of the experiment.

2.2.1) Either in a clear open field box or an enclosed area, mount a video camera from below or above, respectively, to record the pup as it moves around the field. Use gentle prodding by touching the pup's tail to motivate the pup to walk. Record for two mins.

2.2.2) Using the video recordings, measure the foot angle of the pups by drawing a line from the end of the heel/shin to the tip of the longest (middle) toe. Only take the measurement when the pup is performing a full stride in a straight line and both feet are flat on the ground. Do not take measurements while the pup is stationary or while the pup is turning.

2.2.3) Measure three to five sets of foot angles and calculate the average angle for each pup tested.

2.3 Surface Righting (Figure 3):
NOTE: The righting reflex is the motor ability for a mouse pup to be able to flip onto its feet from a supine position. The average age for the righting reflex to appear in rodents is PND 5 with a range from PND 1-10 [15]. As this test is a reflex, there is no learning component and it can be repeated throughout the experimentation period.

2.3.1) Place pups on their backs on a cotton sheet or bench pad and hold in position for five secs.

2.3.2) Release the pups and record the time it takes the pup to return to prone position, as well as the direction of righting (left or right). A total of one min is given for each trial, if needed.

2.3.3) Repeat for a total of three trials.

2.4 *Negative Geotaxis* (Figure 4)**:**
NOTE: The average age for negative geotaxis reflex to appear in rodents is PND 7 with a range from PND 3-15 [15]. The negative geotaxis test assesses motor coordination in young mice. Mice are placed facing down a slope and, due to vestibular cues of gravity, pups turn to face up the slope. The response to stimulus, or taxis, is an innate behavior.

2.4.1) Place the pup with its head pointing downward on a 45° incline and hold it for 5 secs.

2.4.2) Release the pup and record the time and direction the pup turns to face upward. Total testing time is 2 mins.

2.4.3) Repeat for a total of three trials. Mice that fall down the incline or fail to turn can be either re-tested, eliminated, or given a zero score.

NOTE: This decision is left to the examiner, as occasionally pups will roll down the incline due to sleepiness rather than weakness. Once the decision is made on how to score pups that fall down the incline, it should be noted in the methods and should be consistent throughout the testing of all subjects.

2.5 ***Front-limb Suspension*** (Figure 5) [16]; adapted from [17, 18] :
NOTE: Front-limb suspension tests the forelimb strength of pups, including arm and paw strength. This test is not recommended for pups younger than PND 10 [15]. Pups are allowed to grasp a wire strung across a stable object and hang onto the wire with both forepaws. The testing area is over a padded drop zone. The test can detect right/left side strength differences. Learning and the absence of negative reinforcement can lead to increased non-participation. Mice falling immediately when released or failure to grasp when placed on the wire are indicative of non-participation.

2.5.1) Hold the pups firmly by the body and enable them to grasp the wire with both forepaws.

2.5.2) Release the pup. Using a timer or stopwatch, record the total time to fall, as well as paw weakness.

NOTE: Paw weakness is determined if a pup consistently falls from the wire with one paw before the other rather than releasing from the wire with both paws at the same time.

2.5.3) Repeat test for a total of three times.

2.6 ***Hindlimb Suspension*** (Figure 6):
NOTE: This suspension test determines hindlimb strength. It is a test designed specifically for neonates and was initially used on animals between PND 2-12 [19,20], but can be adapted for mice up to PND 14. This test can detect right/left hindlimb strength differences as well as neuromuscular function. A standard 50ml conical is used, padded with laboratory wipes. Similar to the front-limb suspension test, this test can be learned, especially since there is no negative consequence to falling. Thus, increased non-participation, as seen by mice falling as soon as released or failure to stay when placed on the edge of the tube, may be noted.

2.6.1) Using a 50ml conical, place pup gently face down into the tube with its hind legs hung over the rim.

2.6.2) Release the pup. Observe the hindlimb posture.

2.6.2.1) Score posture according to the following criteria.

NOTE: Score of 4 indicates normal hindlimb separation with tail raised; score of 3 means weakness is apparent and hindlimbs are closer together but they seldom touch each other; score of 2 indicates hindlimbs are close to each other and often touching; score of 1 shows a weakness is apparent and the hindlimbs are almost always in a clasped position with the tail raised; a score of 0 indicates constant clasping of the hindlimbs with the tail lowered or failure to hold onto the tube for any period of time.

2.6.2.2) Count pulls if necessary. A pull is qualified when the pup attempts to lift its body using its hindlimbs while suspended on the side of the conical tube.

2.6.3) Using a timer or stopwatch, record the latency to fall.

2.6.4) Repeat the entire test in triplicate.

2.7 *Four Limb Grip Strength* (Figure 7)**:**
NOTE: This test will examine the paw strength of all four paws at the same time. A 16x18 fiberglass screen wire is used. The average age for a rodent to be able to grasp a horizontal screen is PND 8 with a range from PND 5-15 [15]. Fox used the four limb horizontal screen test from PDN 2-21 [10]. This test is modified from the standard horizontal screen test; here the screen is rotated slowly from a horizontal to vertical position, to challenge the grasping of all four limbs [21]; adapted from Corti S [16]. If the mouse holds on to the mesh screen when inverted to 180°, record the latency to fall. Also, note the body weight. A hanging impulse can be calculated as [weight (g) x latency to fall (sec)] reflecting the force needed to resist gravity.

2.7.1) Using a piece of wire mesh, place the pup on the screen. Allow the pup to adjust to this environment for approximately 5 secs.

2.7.2) Invert the screen slowly to 180 degrees. Record the approximate angle of the screen when the pup falls off.

2.7.3) Repeat for a total of three trials and average the trials.

2.8 *Grasping Reflex* (Figure 8)**:**
NOTE: The grasping reflex usually appears in rodents at PND 7 with a range from PND 3-15 [15]. Each paw is tested individually, thus the test can reveal front- or hindlimb issues, as well as sidedness issues. As it is a reflex, this test can be repeated until the reflex appears. It is not prone to learning. As an important caveat, this test does not distinguish grasping strength, only ability, and must be tested prior to 15 days of age when juvenile mice begin to grasp due to fear response.

2.8.1) Hold the mouse by the scruff of its neck, similar to the way a mouse pup is carried by the dam. This hold causes the pup to become instinctively immobile and relaxed, allowing for ease of testing.

2.8.2) Stroke each paw of the pup with the blunt, rounded side of a razor blade.

2.8.3) Test each paw individually and record the presence or absence of grasping and score 1 point per paw with which the mouse grasps.

NOTE: The scoring for right paw preference is 100% for right paw preference, -100% for left paw preference, 50% for both paws grasping, and 0% for no paws grasping. The equation to determine these numbers is [(right paw – left paw)/(right paw + left paw + both paws)]*100%.

2.9) *Cliff Aversion* (Figure 9):
NOTE: Cliff aversion tests labyrinth reflexes, as well as strength and coordination and can be used to test pups from PND1-14 [22]. A pre-scented box (a box where a minimum of 5 mice have been allowed to freely roam) with a flat elevated ledge is used and the pup is placed with the digits only of their forepaws and their snout positioned over the edge. Scoring is performed by counting the total time it takes the pup to turn away from the cliff and move its paws and snout away from the edge. If no response is seen after 30 secs, the test is terminated. If the pup falls off the edge, a single additional trial can be performed.

2.9.1) Using a side view, place the pup on the edge of the pre-scented box, making sure that the digits and snout are the only parts over the edge.

2.9.2) Release pup and start timer.

2.9.3) Once both the snout and paws have been removed from the edge, stop the timer and record time.

2.9.4) Repeat test for a total of 3 trials. If the pup does not move away from cliff within 30 secs, no score is given. NOTE: The determination whether the pup is a non-participator versus impaired is left up to the discretion of the examiner.

3. Statistical Significance
3.1) Using a statistical software analyze the results. Express the data as mean ± standard error of the mean (SEM). Tests are parametric and thus, examine the data using t-test analyses.

NOTE: Experiments were not designed to test for gender differences. Differences are considered to be statistically significant when $p<0.05$.

REPRESENTATIVE RESULTS:
Mice were tested from P7 (24 hours following surgery) to P13 (1 week following surgery), using different mice for each time-point so that learning a testing paradigm was not a confounding variable. P8 was selected as representative results, as mice showed the greatest deficits at this time point.

Transition from crawling to walking is delayed in HIL neonatal mice

Human CP patients have gait abnormalities, ranging from toe-walking to a scissored gait. As this CP model displays gait deficits similar to humans, ambulation was assessed. Mice were scored on gait symmetry and limb-paw movement during a straight walk. At 48 hrs following surgery (PND 8), HIL mice had less symmetric limb movement and a "crawling" gait as compared to their sham counterparts (average ambulation score: HIL 1.083 ± 0.6337, n = 12 vs sham 1.639 ± 0.4859, n = 9; $p<0.05$, Figure 10). By one week, both HIL and sham mice have transitioned to walking (data not shown).

Hindlimb foot angle is increased in HIL

In addition to ambulation, hindlimb foot-angle was assessed. Eight-day-old sham mouse pups walk with their hindpaws facing forward, compared to HIL mice, who have splayed hindpaws when walking in a straight line (Figure 2; average angle: HIL 77.48 ± 9.848, n = 9, vs sham 54.54 ± 8.043, n = 11; $p<0.0001$, Figure 11). This increased angle correlates with gait instability, in that the pups need to increase the angle of their rear paws in order to stabilize their gait and assist with balance and coordination.

HIL mice do not show deficits when surface righting

The surface righting test was included as some CP patients have impaired trunk control (Heyrman et al., 2013). Additionally, the vestibular system is necessary to detect the need for righting and there are vestibular deficits in some CP patients [23]. HIL mice do not show significant deficits when righting as compared to sham controls (data not shown).

HIL mice perform the same as sham in negative geotaxis testing

Negative geotaxis is used to test motor coordination in young pups. Mice are challenged by being place facing downhill on a sloped surface. Delay or failure to orient uphill could indicate deficits in coordination, balance, or vestibular input. HIL mice show no deficits when challenged with negative geotaxis as compared to sham mice (data not shown). Additionally, HIL mice did not show a preference to turn toward one side versus another when re-orienting.

Front-limb suspension test is appropriate for mice older than 10 days

CP patients have decreased muscle tone and deficits in fine motor skills, such as grasping. To test weakness in this mouse model, we used a front-limb suspension test. Furthermore, this model uses unilateral ischemic injury and sided-ness could be determined using this suspension test. This test is better for mice older than 10 days [15]. At 8 days old, two days following injury, there were no significant differences between HIL and sham mice (data not shown).

Hindlimb strength is decreased in HIL mice

Human CP patients often need braces or assistive walking devices due to lack of motor control and strength. In order to compare the rodent CP model to humans, hindlimb strength was assessed using the hindlimb suspension test. When suspended from the side of a conical tube, HIL mice showed hindlimb weakness, as demonstrated by a decrease in hanging score (hindlimb hanging score: HIL 3.468 ± 0.5561, n = 13, vs sham 3.891 ± 0.1329, n = 13; $p<0.05$, Figure 12). No difference was observed in hindlimb suspension time (data not shown). Thus, similar to human CP patients, HIL mice demonstrate hindlimb (leg) weakness.

Four limb grip strength is decreased following HIL injury

Grasping with all four paws is important for a rodent in terms of climbing and running across uneven surfaces. Four limb grip requires significant sustained strength, rather than dexterity or linear force, mainly in the digits and paws [24]. Mice were required to hold their body weight on an inverted wire mesh screen. HIL mice were not able maintain their grip and these mice fell at significantly lower angles (four limb average angle: HIL 75.627 ± 24.48, n = 11, vs sham 96.57 ± 10.836, n = 9; $p<0.05$, Figure 13). This data shows that there is a significant deficit in grip strength in HIL mice.

Grasping reflex deficits are apparent in HIL mice

Along with gross motor deficits, fine motor movements are also impaired in CP patients [25,26]. The grasping reflex in humans is present at birth and disappears around 5-6 months. However, changes in the grasping reflex, such as exaggerated velocity or strength of grasping, failure to grasp, or the reemergence of the grasping reflex after 6 months of age, all indicate damage to the nervous system. To compare grasping in the CP model, reflexive grasping deficits were determined.

At 48 hrs after injury, HIL mice demonstrate a decrease in grasping reflex (average paws grasped at 48 hrs: HIL 2.429 ± 0.9376, n = 14, vs sham 3.214 ± 0.8018, n = 14; $p<0.05$, Figure 14a). There was a slight, but not significant increase in right paw preference in the forepaws (data not shown). There was a significant right paw preference in the hindpaws (HIL 75.0 ± 42.74, n = 14, vs sham 17.86 ± 54.09, n = 14; $p<0.005$, Figure 14). One week after injury, HIL mice show grasping deficits (average paws grasped at 1 week: HIL 2.75 ± 1.035, n = 8, vs sham 3.80 ± 0.6325, n = 10; $p<0.05$, Figure 14c), with no notable paw preference.

HIL mice turn away from the edge during cliff aversion

The cliff aversion test relies on the inherent fear of the mice to turn away from a steep cliff and head towards safety. Although some CP patients have vestibular difficulties, as well as impaired motor control, the HIL mice did not show any deficits on this test.

FIGURE LEGENDS:

Figure 1. The transition from crawling to walking can be distinguished by observing the hindpaw, as well as the head and tail. A. During crawling, the entire back paw, from the toes to the heel, touches the ground when ambulating, as denoted by (*). An adult walking pattern is seen when only the toes and front part of the hindpaw touch the ground (the heel is elevated, deonoted by [**]). B. The head and tail of a crawling mouse is low to the ground. The head begins to rise during the transition from crawling to walking. The transition is complete when both the head and tail are elevated and only the front of the hindpaw touches the ground.

Figure 2. Hindlimb foot angle can be used to determine gait abnormalities. The foot angle can be measured by drawing a line from the mid-heel through the middle (longest) digit. Injured animals have a greater foot angle when compared to normal (see Representative Results, Foot Angle).

Figure 3. Surface righting. This test requires trunk control and may test for postural imbalances. Human CP patients may have deficits in their core.

Figure 4. Negative geotaxis. Motor and vestibular input is required for the mouse to recognize its orientation on a slope and turn around.

Figure 5. Front-limb suspension. This suspension test causes tension in the forelimbs until muscle fatigue. With this approach, baseline strength in the forelimbs are established.

Figure 6. Hindlimb suspension. A. This suspension test causes tension in the hindlimbs until muscle fatigue. Baseline strength and posture in the hindlimbs are established. B. Scoring. Note the numbers above the representative mice demonstrating the possible posture score.

Figure 7. Four limb grip strength. Mice are required to sustain muscle tension in all four limbs as gravitational force increases.

Figure 8. Grasping reflex. Because neonatal mice do not have a strong fear response, this test strictly determines the plantar/palmar reflex.

Figure 9. Cliff aversion. Vestibular imbalances are measured using the cliff aversion test. Here, the pup's eyes are still closed so fear is not the driving factor to turn away from the cliff's edge.

Figure 10. HIL mouse pups do not ambulate as well as shams. Sham mice (black bar) have a mean score of 1.639 ± 0.4859 (n = 9), meaning their ambulatory development falls between asymmetric limb movement and slow crawling. HIL mice (gray bar) receive an average score of 1.083 ± 0.6337 (n = 12), meaning their ambulation is less developed and tend to have asymmetric limb movement. Data are expressed as mean ± SEM; * is $p<0.05$.

Figure 11. HIL mouse pups splay their hindpaws when walking. HIL mice (black bars) have an average angle between their hindlimbs of 77.48 ± 8.043 (n = 11), while sham mice (gray bars) have an average angle of 54.54 ± 9.848 (n = 9). Data are expressed as mean ± SEM; **** is $p<0.0001$.

Figure 12. Sham mice are slightly but significantly stronger in their hindlimbs than HIL mice. At an average hanging score of 3.891 ± 0.1329 (n = 13), sham mice (black bar) show more hindlimb separation, and therefore a stronger hindlimb stance, when hanging on the edge of a tube than HIL mice (gray bar) with an average hanging score of 3.468 ± 0.5561 (n = 13). Data are expressed as mean ± SEM; * is $p<0.05$.

Figure 13. HIL mice have weaker four limb grip than shams. Sham mice (black bar) can grasp to an average inverted angle of 96.57 ± 10.836 (n = 9). HIL mice (gray bar) can only reach an inverted angle of 75.627 ± 24.48 (n = 11). Data are expressed as mean ± SEM; * is $p<0.05$.

Figure 14. HIL mice have grasping deficits, in the hindpaws, contralateral to the injured brain region. A) 48 hrs following injury (PND 8), HIL mice (gray bar) grasp a stick with, on average, fewer paws than sham animals (black bar). B) HIL mice (gray bar) display a preference for grasping with the right hindpaw (contralateral to injury) as opposed to using the left hindpaw (ipsilateral to injury). Sham mice (black bar) do not display this right paw preference. Right paw preference is calculated as ([right paw - left paw]/[right paw + left paw + both paws] * 100). C) One week following injury, HIL mice (gray bar) still show grasping deficits as compared to shams (black bar). Data are expressed as mean ± SEM; * is $p<0.05$, ** is $p<0.005$.

DISCUSSION:

Using animal models to study human diseases is only relevant if there is overlap between the cellular and molecular response between human and rodent and that the behavioral tests performed have direct relevance to human symptoms. One of the major issues with pediatric disease studies is that many researchers use adult rodents to create the model, as well as adult rodent behavioral evaluation, without considering the developmental differences that may be important for the disease process. Because of these issues, it is important that research on pediatric disease use not only the appropriate adjusted developmental time-points (e.g. human CNS development at 28-32 weeks is equivalent to a post-natal day 2-7 day rodent) [7], but also behavioral tests that will examine appropriate motor, sensory or reflexive developmental behaviors. Thus, as each new neonatal disease model is developed, it must be rigorously tested to assure that the cellular and behavioral responses will provide the most appropriate translatable data between rodent and human.

Cerebral palsy is a motor disorder, which persist into adulthood. One problem with many of the cerebral palsy models available today is the lack of repeatable, standardized motor testing that can correlate with the deficits seen in pediatric patients. In this new model, which combines hypoxia, ischemia and inflammation in a neonatal mouse, motor behavior was evaluated using a battery of tests specific for neonatal mice. In order to decrease the subjectivity and increase the quantitative reporting, several tests have been modified to include very specific, but easy to evaluate measures that can be standardized. In addition, front- and hindlimb evaluations can be performed separately, and left/right differences can be determined. This battery of tests is specific for neonatal mice up to two weeks of age.

This CP model demonstrates difficulty in walking (ambulation, hindlimb foot angle), as well limb-specific weakness (four limb suspension, hindlimb suspension), and deficits in developmental reflexes (grasping reflex). Although in this study only one timepoint was examined, these deficits can be tracked over time.

There are other batteries of tests that can be used on the neonate, such as the Fox's battery of tests or Heyser's Assessment of Developmental Milestones [15]. However, these tests compare the neonate to the adult, whose responses may not be the same because the neonate is still developing. Fox's battery and Heyser's Assement tests rely on observational subjective information with dichotomous (yes or no) assessment, rather than objective data (angle, posture based on strength, etc). Because of the subjectiveness of these tests, many scientists

have adapted, added, or removed criteria, thus making their results incomparable to others and limiting the usefulness of the data in terms of establishing a baseline deficit for a particular disease or disorder. By establishing one set of standardized motor tests that are qualitative and specifically designed to test neonates, results from individual research groups can be accurately and reliably reported and compared.

ACKNOWLEDGEMENTS:
We would like to thank everyone at Shriners Hospital Pediatric Research Center, in particular Dr. Mickey Seltzer, of whom without his support, this work would not have been funded. In addition, we would like to thank Isha Srivastava, who contributed to early data collection and Amy He, who helped with the figures. This study was funded by Shriners Hospitals for Children. No funding source played a role in experimental design or decision to submit the paper for publication.

DISCLOSURES:
The authors declare that they have no competing financial interests.

REFERENCES:
1. Nakamura, M. & Bregman, B. S. Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. *Exp Neurol* **169** (2), 407–415, doi:10.1006/exnr.2001.7670 (2001).
2. Widenfalk, J., Lundströmer, K., Jubran, M., Brene, S. & Olson, L. Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. *J Neurosci* **21** (10), 3457–3475 (2001).
3. Fernández-López, D., Faustino, J., *et al.* Blood-brain barrier permeability is increased after acute adult stroke but not neonatal stroke in the rat. *J Neurosci* **32** (28), 9588–9600, doi:10.1523/JNEUROSCI.5977-11.2012 (2012).
4. Cusick, C. G. Extensive cortical reorganization following sciatic nerve injury in adult rats versus restricted reorganization after neonatal injury: implications for spatial and temporal limits on somatosensory plasticity. *Prog Brain Res* **108**, 379–390 (1996).
5. Barrett, C. P., Donati, E. J. & Guth, L. Differences between adult and neonatal rats in their astroglial response to spinal injury. *Exp Neurol* **84** (2), 374–385 (1984).
6. Villapol, S., Gelot, A., Renolleau, S. & Charriaut-Marlangue, C. Astrocyte Responses after Neonatal Ischemia: The Yin and the Yang. *Neuroscientist* **14** (4), 339–344, doi:10.1177/1073858408316003 (2008).
7. Craig, A., Ling Luo, N., *et al.* Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp Neurol* **181** (2), 231–240 (2003).
8. Shen, Y., Liu, X.-B., Pleasure, D. E. & Deng, W. Axon-glia synapses are highly vulnerable to white matter injury in the developing brain. *J Neurosci Res* **90** (1), 105–121, doi:10.1002/jnr.22722 (2012).
9. Shen, Y., Plane J. M. & Deng, W. Mouse models of periventricular leukomalacia. *J Vis Exp* **18** 39, doi:10.3791/1951 (2010).
10. Fox, W. M. Reflex-ontogeny and behavioural development of the mouse. *Anim Behav* **13** (2), 234–241 (1965).

11. Tremml, P., Lipp, H. P., Müller, U., Ricceri, L. & Wolfer, D. P. Neurobehavioral development, adult openfield exploration and swimming navigation learning in mice with a modified beta-amyloid precursor protein gene. *Behav Brain Res* **95** (1), 65–76 (1998).
12. Wahlsten, D. A developmental time scale for postnatal changes in brain and behavior of B6D2F2 mice. *Brain Res* **72** (2), 251–264 (1974).
13. Balasubramaniam, J., Xue, M. & Del Bigio, M. Long-term motor deficit following periventricular hemorrhage in neonatal rats: A potential model for human cerebral palsy. *J Cerebr Blood F Met* (2005).
14. Williams, E. & Scott, J. P. The Development of Social Behavior Patterns in the Mouse, in Relation to Natural Periods. *Behaviour* **6** (1), 35–65 (1954).
15. Heyser, C. J. Assessment of developmental milestones in rodents. *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.]* **Chapter 8**, Unit 8.18, doi:10.1002/0471142301.ns0818s25 (2004).
16. Corti, S. TREAT-NMD : Experimental protocols for SMA animal models. *treat-nmd.eu* at <http://www.treat-nmd.eu/research/preclinical/sma-sops/>
17. Corti, S., Nizzardo, M., *et al.* Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. *J Clin Invest* **118** (10), 3316–3330, doi:10.1172/JCI35432 (2008).
18. Grondard, C., Biondi, O., *et al.* Regular exercise prolongs survival in a type 2 spinal muscular atrophy model mouse. *J Neurosci* **25** (33), 7615–7622, doi:10.1523/JNEUROSCI.1245-05.2005 (2005).
19. El-Khodor, B. F. Behavioral Phenotyping for Neonates. *Experimental Protocols for SMA animal models* at <http://www.treat-nmd.eu/research/preclinical/sma-sops/>
20. El-Khodor, B. F., Edgar, N., *et al.* Identification of a battery of tests for drug candidate evaluation in the SMNDelta7 neonate model of spinal muscular atrophy. *Exp Neurol* **212** (1), 29–43, doi:10.1016/j.expneurol.2008.02.025 (2008).
21. Venerosi, A., Ricceri, L., Scattoni, M. L. & Calamandrei, G. Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in CD-1 mouse pups. *Environ Health* **8**, 12, doi:10.1186/1476-069X-8-12 (2009).
22. Hill, J. M., Lim, M. A. & M, S. M. *Neuromethods*. (Humana Press: New Jersey, 2008).
23. Yu, Y., Keshner, A. E., Tucker, C. A., Thompson, E. D. & Lauer, R. T. Visual dependence influences postural responses to continuous visual field motion in adults with cerebral palsy. (2015).
24. *Experimental Protocols for SMA animal models*. at <http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.1.005.pdf> (2011).
25. Gordon, A. M. & Duff, S. V. Relation between clinical measures and fine manipulative control in children with hemiplegic cerebral palsy. *Dev Med Child Neurol* **41** (9), 586–591 (1999).
26. Futagi, Y., Toribe, Y. & Suzuki, Y. The grasp reflex and moro reflex in infants: hierarchy of primitive reflex responses. *Int J Pediat* **2012**, 191562, doi:10.1155/2012/191562 (2012).

Figure 1, Ambulation

Click here to download Figure: Figure 1, Ambulation.pdf

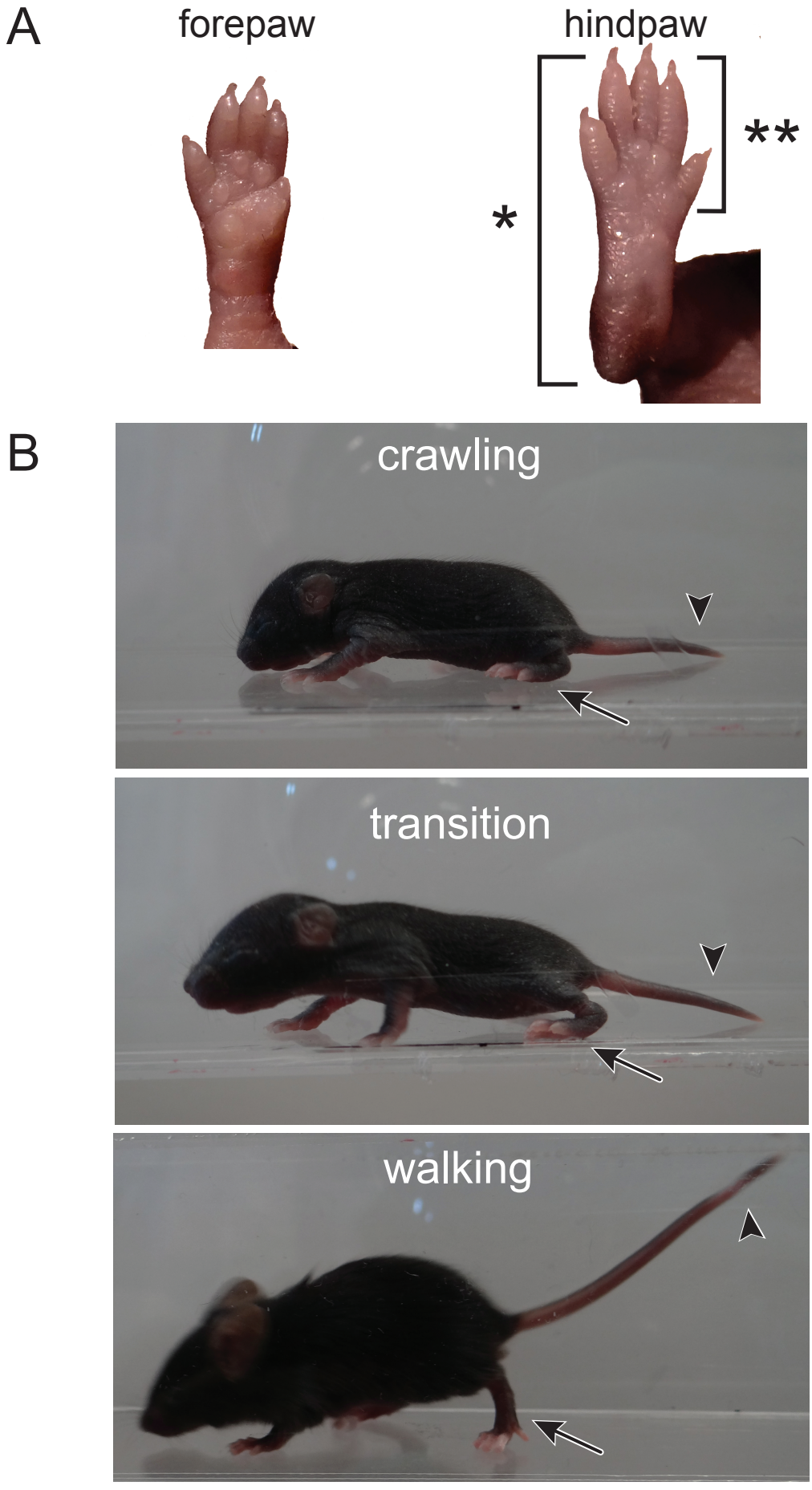


Figure 2, Ambulation Angle
Click here to download Figure: Figure 2, Ambulation Angle.pdf

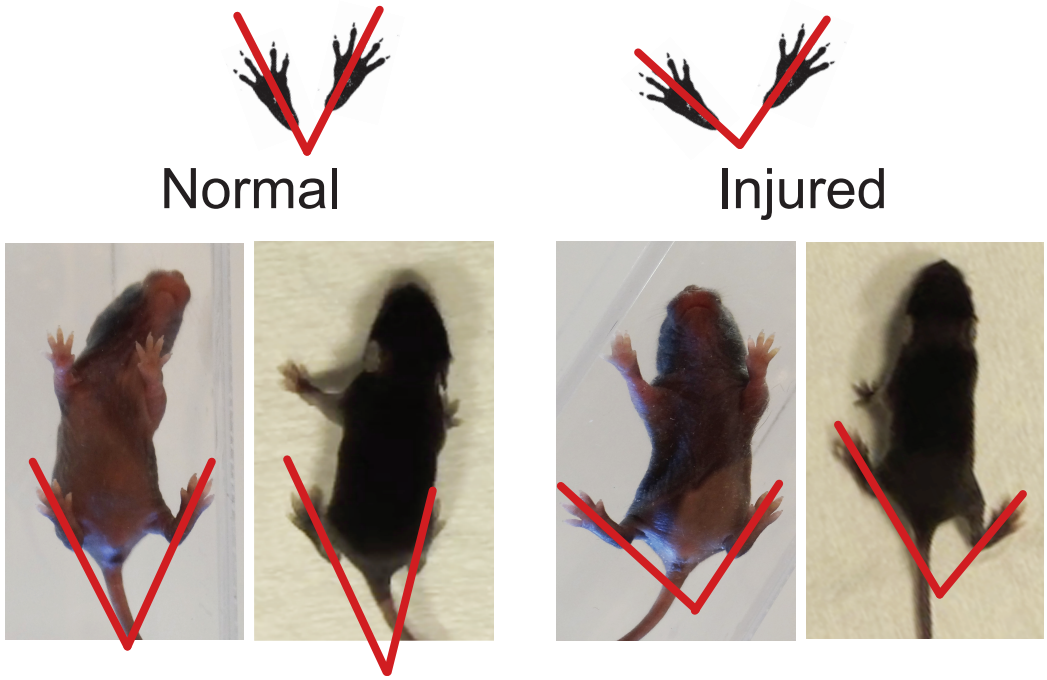


Figure 3, Righting
Click here to download Figure: Figure 3, Righting neonatal.pdf

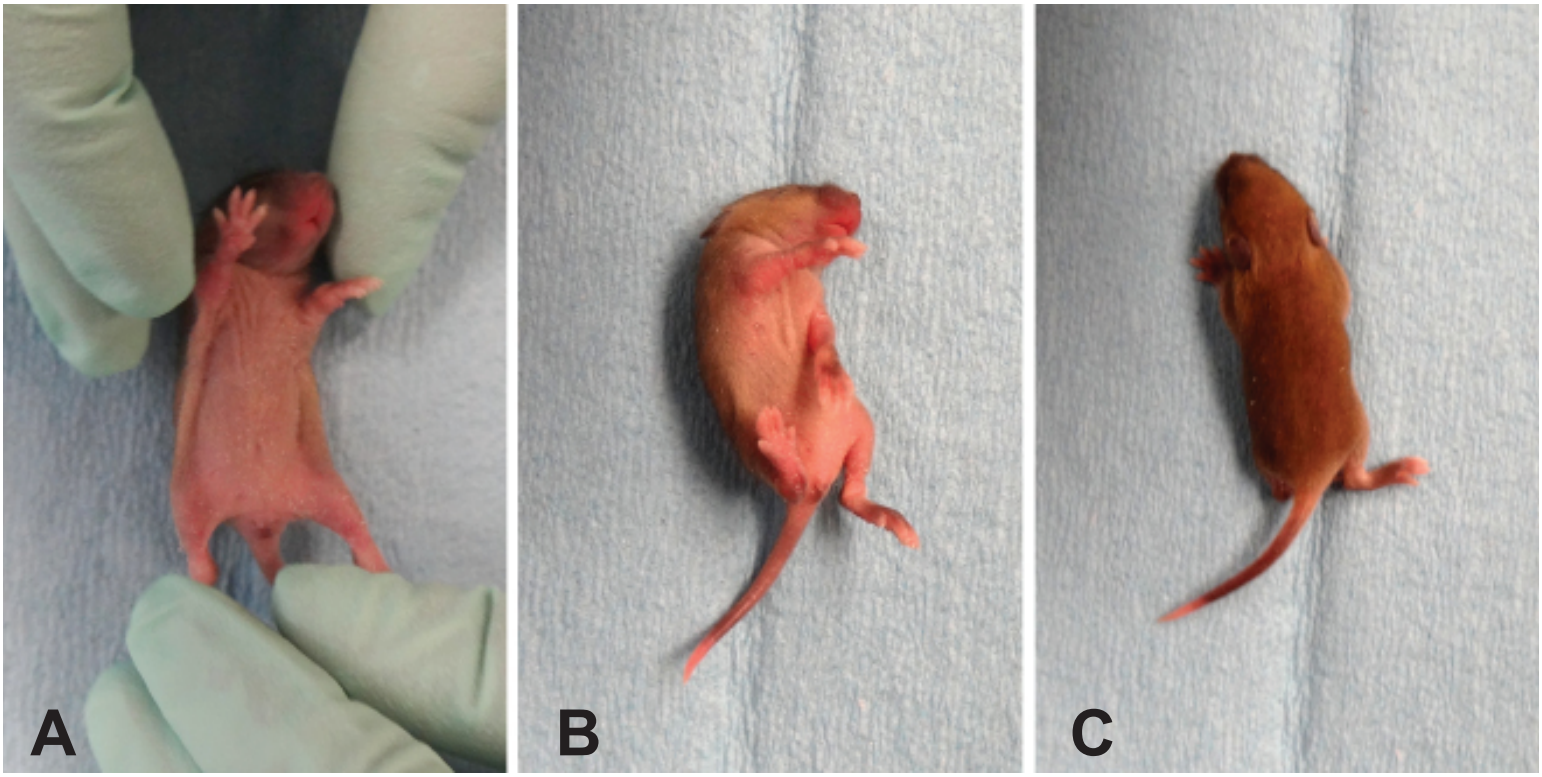


Figure 4, Negative Geotaxis
Click here to download Figure: Figure 4, negative geotaxis.pdf

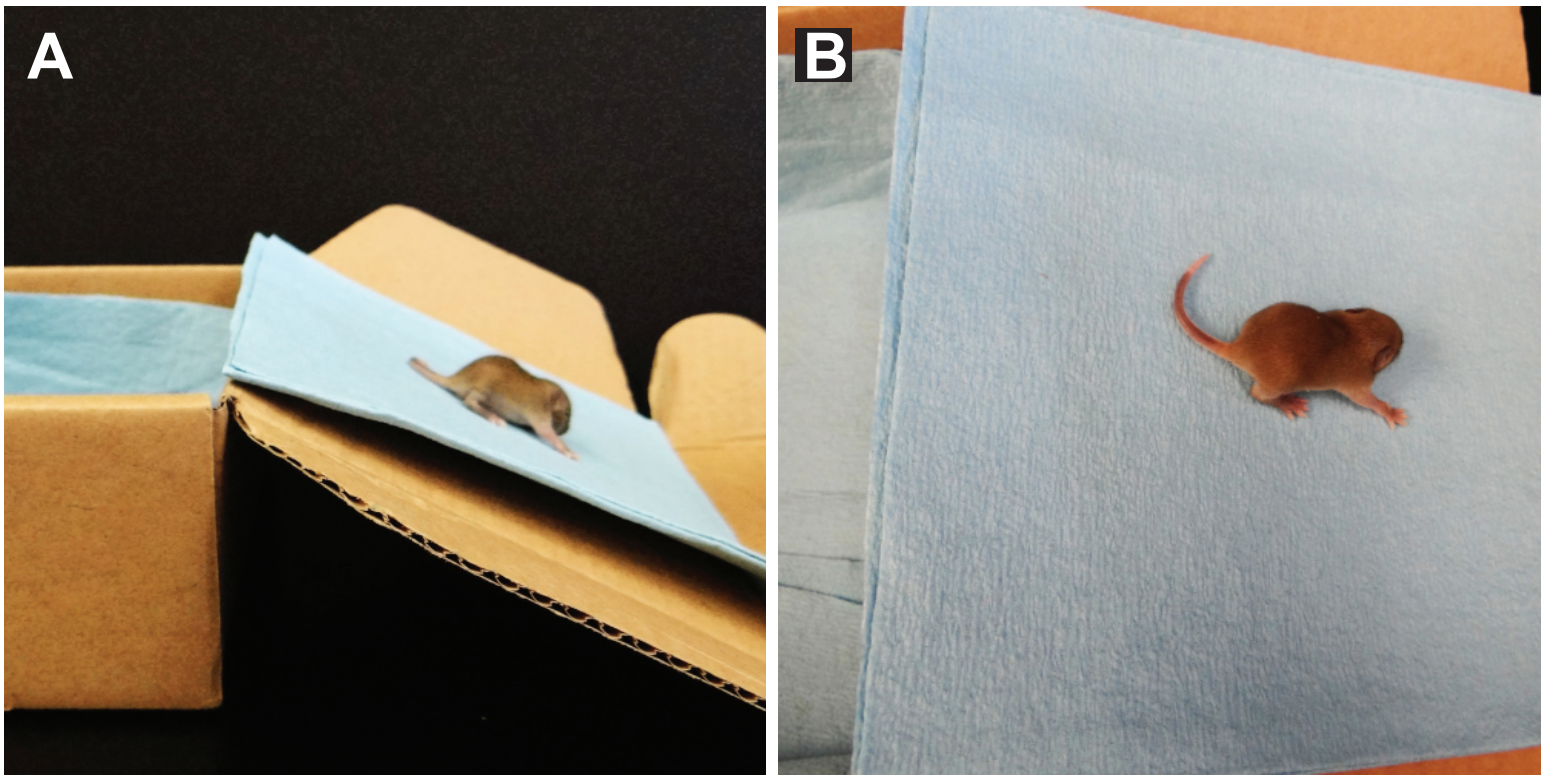


Figure 5, Front Limb Suspension
Click here to download Figure: Figure 5, front limb suspension.pdf

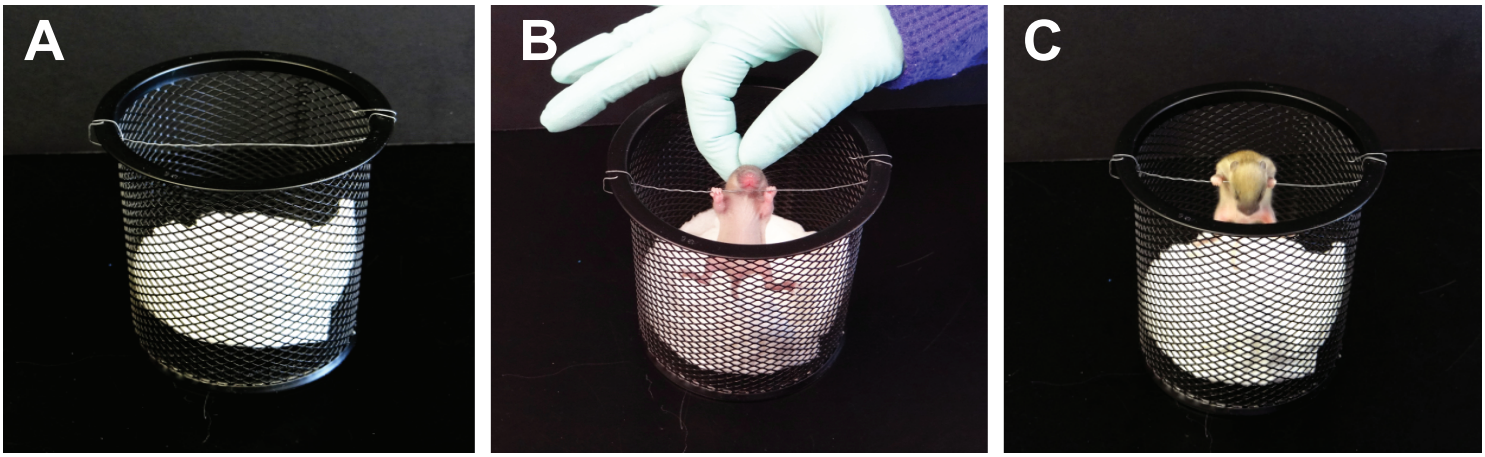


Figure 6
Click here to download Figure: Figure 6, labeled.pdf

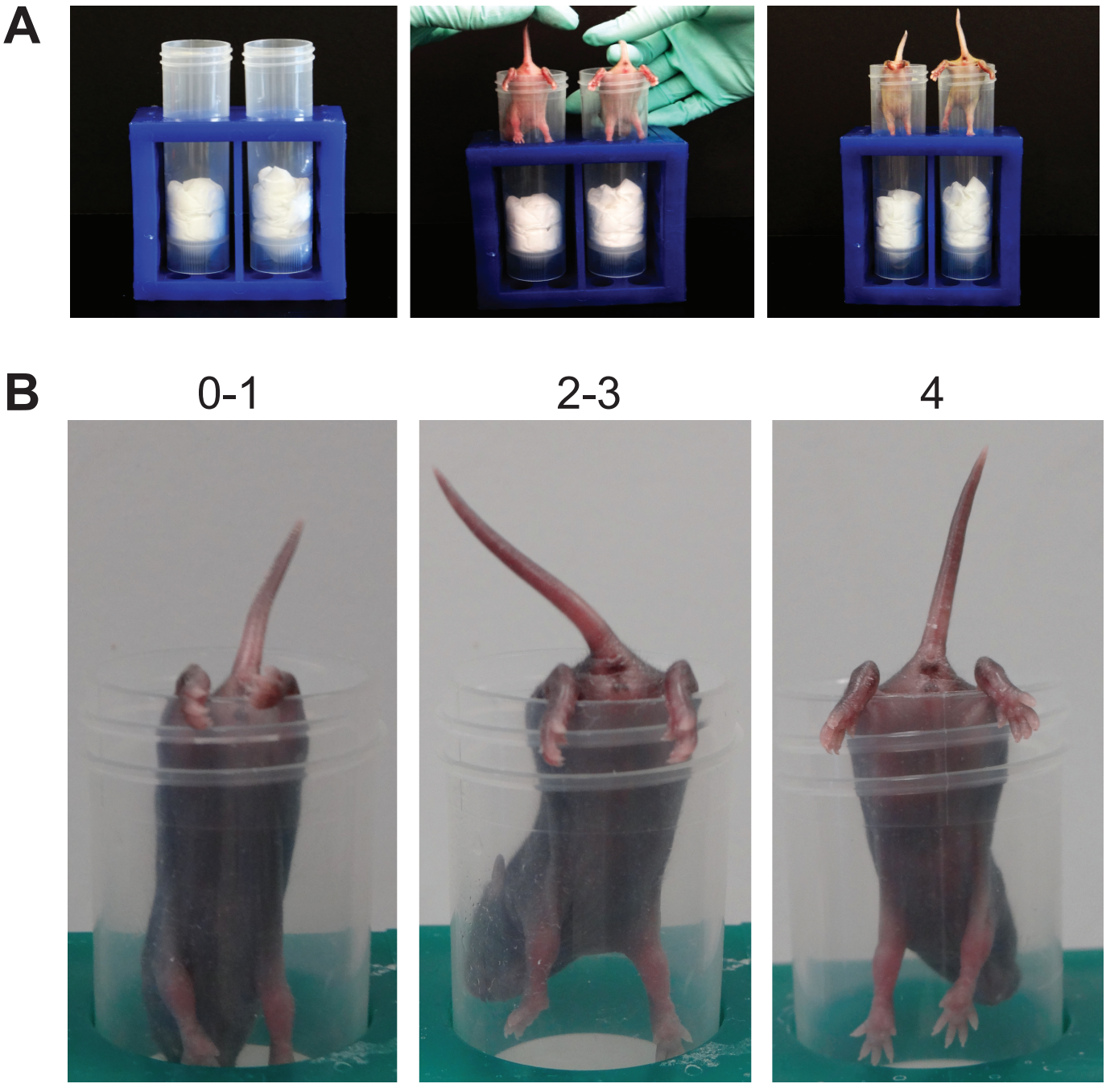


Figure 7, Four Limb Grip
Click here to download Figure: Figure 7, four limb grip strength.pdf

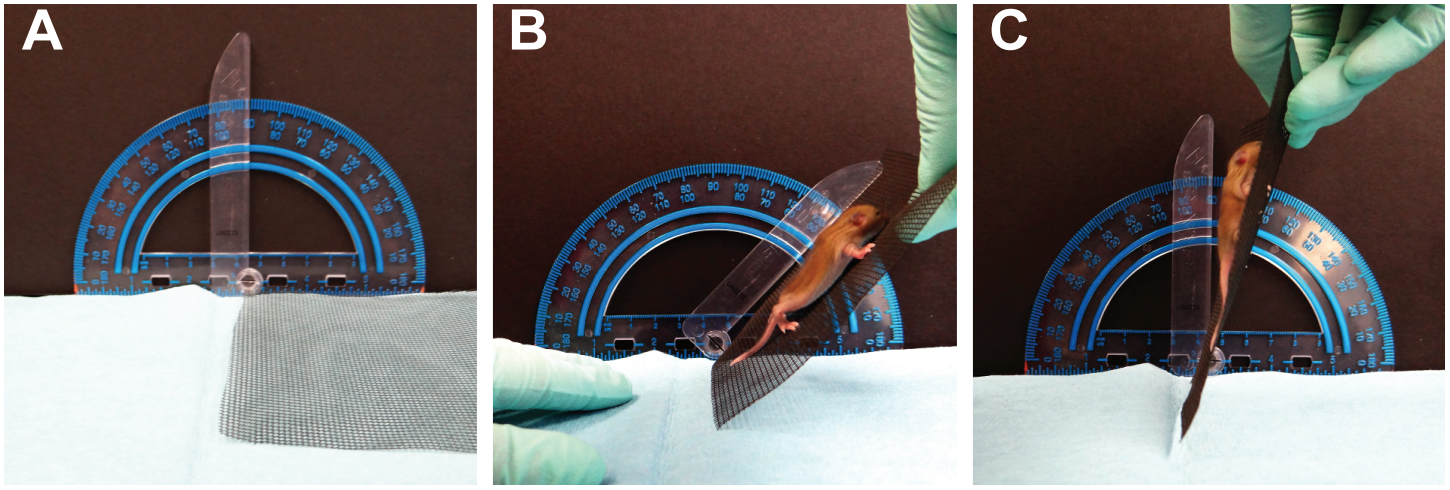


Figure 8, Grasping Reflex
Click here to download Figure: Figure 8, grasping reflex.pdf

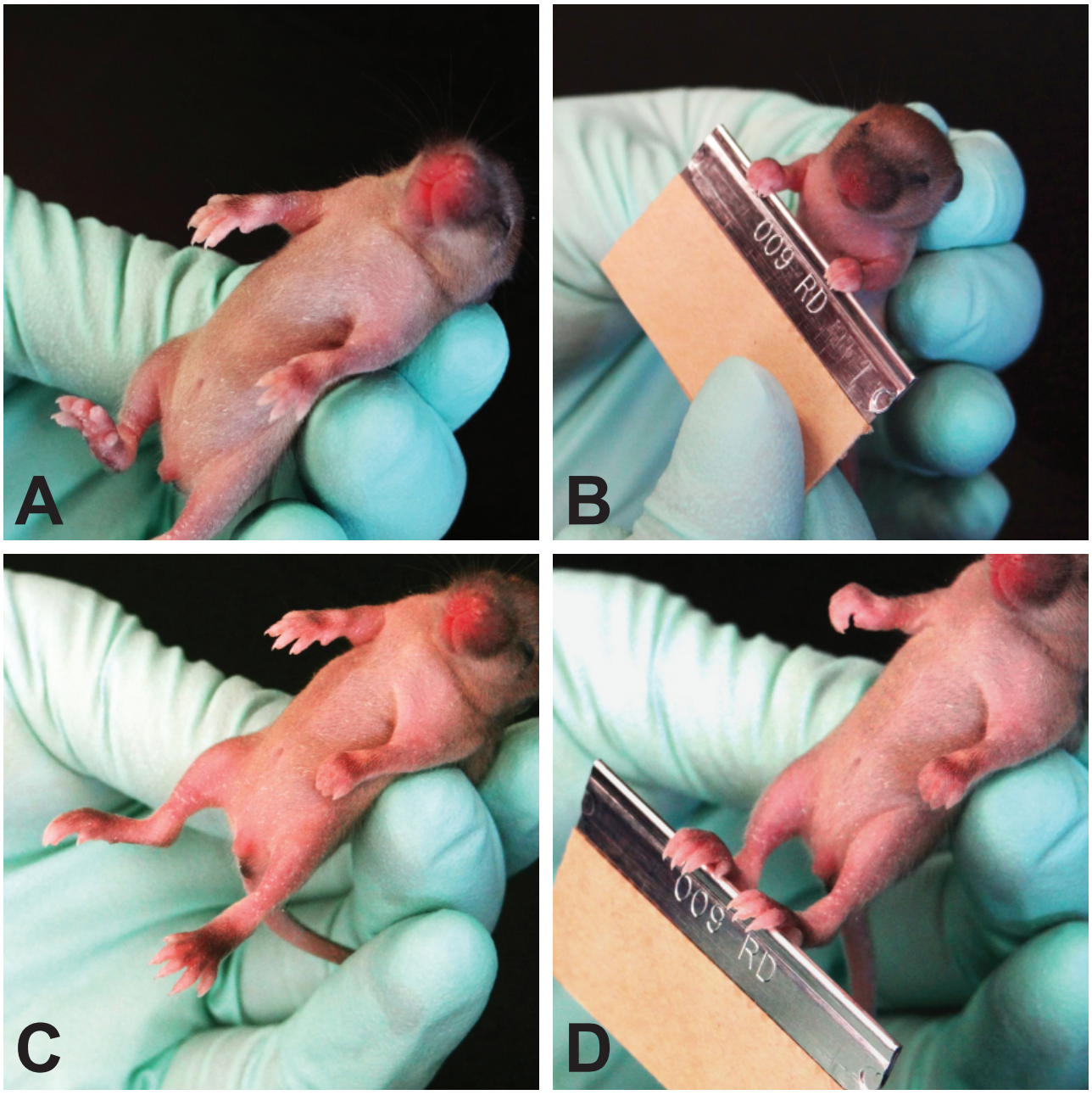


Figure 9, Cliff Aversion
Click here to download Figure: Figure 9, cliff aversion.pdf

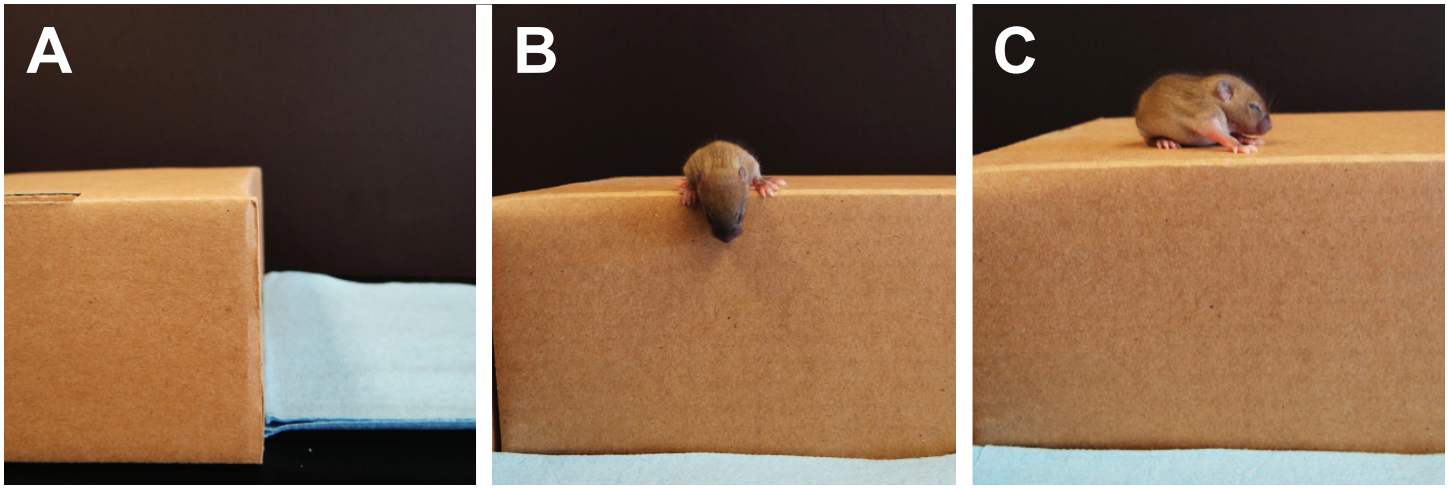


Figure 10, 48 hr Ambulation

Click here to download Figure: Figure 10_ 48hr Ambulation.pdf

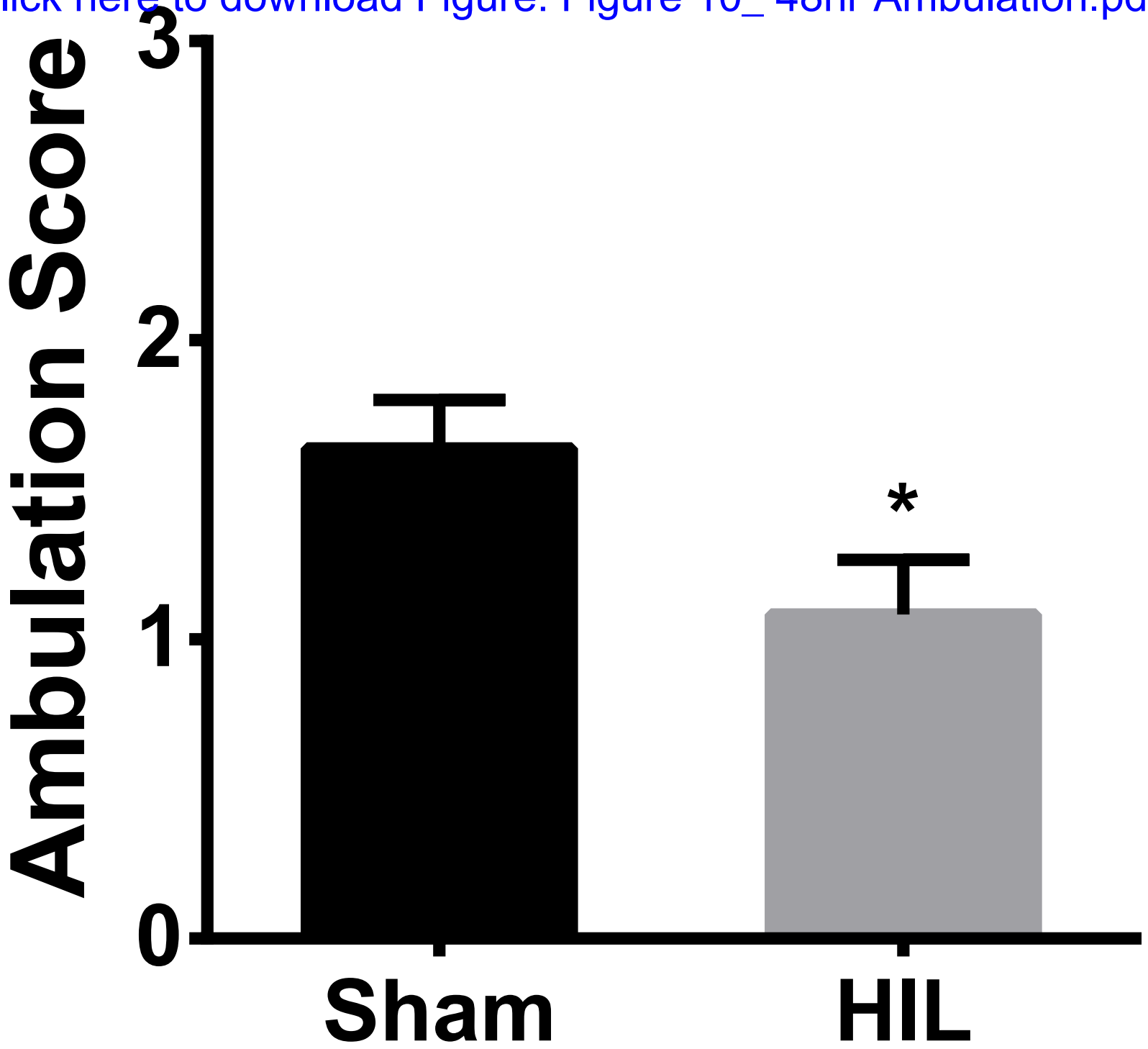


Figure 11, 48 hr Ambulation Angle

Click here to download Figure: Figure 11_ 48hr Ambulation Angle.pdf

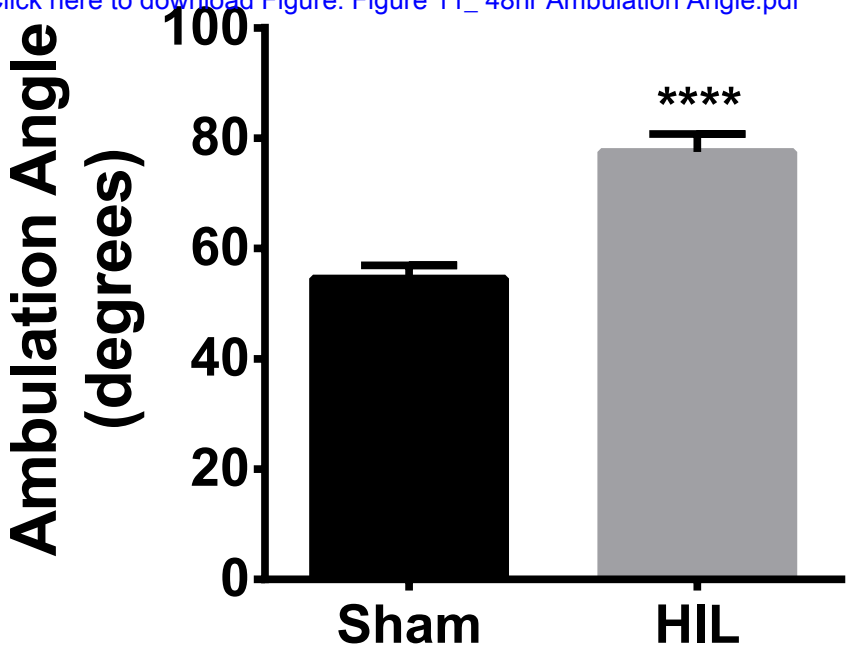


Figure 12, Hind Limb Suspension

Click here to download Figure: Figure 12_ 48hr Hind Limb Suspension.pdf

Figure 13, Four Limb Grip

Click here to download Figure: Figure 13_ 48hr Four Limb Grip ANGLES.pdf

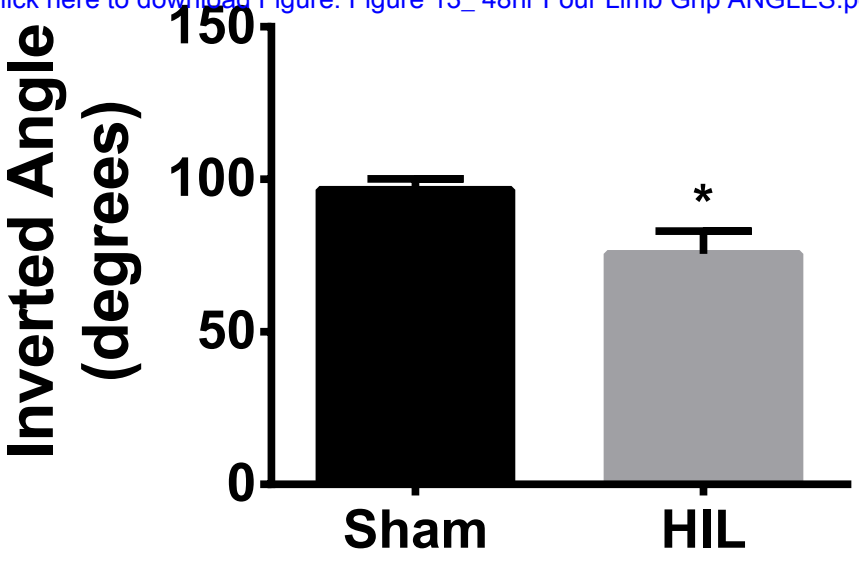
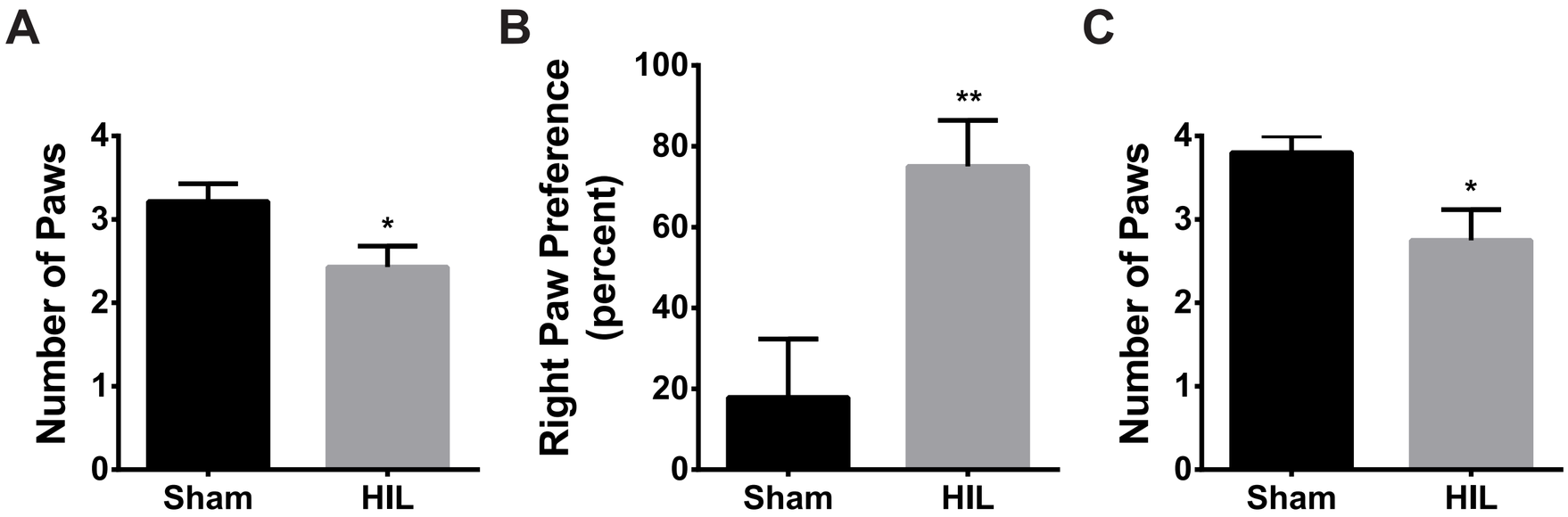


Figure 14, Grasping
Click here to download Figure: Figure 14_ Grasping.pdf



Scoring Sheet
Click here to download Table: Feather-Ferguson Score Sheet.docx

Feather-Ferguson Battery of Neonatal Mouse Motor Tests

Pup ID Number:
Mom ID:
Date Of Birth:
Date Of Behavior Testing:

Initials of investigator performing the test:

Light (lux):
Sound Level:
Temperature:
Humidity:
Start Time:
Finish Time:

1. **Ambulation:** Observe pup for 2 minutes on a solid surface.
   no movement = 0
   slow crawling, asymmetric limb movements = 1
   slow crawling, symmetric limb movement =2
   fast crawling = 3

   Abnormal Movement: Circling: _____ Tremor: _____
   Score 1: _____ **Score 2:** _____ **Score 3:** _____ **Average:** _____

2. **Hindlimb Foot Angle:** Record (from above or below) pup walking and determine hindlimb angle during gait while hindpaws are both flat.

   Degree of hind limb splay: _____ _____ _____ _____ _____ **Average:** _____

3. **Surface Righting:** Place mice on their backs on a cotton sheet. Time to return to prone position and the direction of turning is recorded. Test 3 times. Duration is limited to 2 minutes.
   Score: 0 = No Response 1 = >1 sec 2 = <1 sec

   Trial 1: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 2: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 3: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Average: _____ _____

4. **Negative Geotaxis:** Each pup is placed head downward on an incline and held for 5 seconds. The pup is released and the time and direction to face upward on the incline is recorded. Test 3 times. Duration is limited to 2 minutes.
   Score: 0 = Turn and walk up slope 1 = No turn, slip, no locomotion

   Trial 1: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 2: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 3: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Average: _____ _____

5. **Front-limb Suspension:** Allow pup to grasp a wire with forepaws. Time of falling is recorded. Test 3 times. Record if one paw seems weaker or doesn't grasp as well as the other.

   Trial 1: Time hanging = _____ **Paw Release = LEFT RIGHT BOTH**
   Trial 2: Time hanging = _____ **Paw Release = LEFT RIGHT BOTH**
   Trial 3: Time hanging = _____ **Paw Release = LEFT RIGHT BOTH**
   Average: _____

6. **Hindlimb Suspension:** Gently place pup inside tube face down with its hind legs over the rim of the tube so it is hanging on the side of the tube. Record data including time hanging on tube, number of pulls, and hind limb score. Test 3 times.
   4 = normal hind-limb separation with tail raised
   3 = weakness is apparent and hind limbs are closer together but they seldom touch each other
   2 = hind limbs are close to each other and often touching
   1 = weakness apparent, hind limbs are almost always in a clasped position with the tail raised
   0 = constant clasping of the hind limbs with the tail lowered or failure to hold onto the tube

   Trial 1: Time hanging = _____ **Score =** _____
   Trial 2: Time hanging = _____ **Score =** _____
   Trial 3: Time hanging = _____ **Score =** _____
   Average: _____ **Average:** _____

7. **Four Limb Grip Strength:** Place mouse on wire mesh screen. Allow pup to adjust to environment approximately 5 seconds then begin inverting the screen. Record the approximate angle of the screen when the pup falls off (vertical or horizontal). Test 3 times and average tests. Record neonate body weight and, if the pup hangs on to the screen at 180° for a period of time, determine hanging impulse.

   Trial 1: Screen Position (Circle) = 0° 45° 90° 135° 180°
   Trial 2: Screen Position (Circle) = 0° 45° 90° 135° 180°
   Trial 3: Screen Position (Circle) = 0° 45° 90° 135° 180°
   Average: _____

8. **Grasping Reflex:** Stroke pups paw with the blunt end of a razor blade and determine if mouse can grasp with each paw individually.

   Right Front: _____ **Left Front:** _____
   Right Rear: _____ **Left Rear:** _____

9. **Cliff Aversion:** Pup placed on the edge of a flat surface with snout and forepaws placed over the "cliff". Aversion time is recorded with test duration limited to 30 seconds. Test 3 times.
   Score: 0 = no movement from edge 1 = movement from edge

   Trial 1: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 2: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Trial 3: Direction of Turn = RIGHT LEFT Score: _____ **Time:** _____
   Average: _____ _____

NOTES:

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Table of specific materials and equipment.xls

Name of the Material/Equipment	Company	Catalog Number
C57BL/6 mice	Charles River Laboratories	STRAIN CODE: 027
Anesthesia Dish, PYREX™ Crystallizing Dish	Corning Life Sciences Glass	3140125
Covered lead ring	Fisher Scientific	S90139C
Scalpel Blade #11	World Precision Instrucments, Inc.	500240
Small Vessel Cauterizer	Fine Science Tools	18000-00
Micro Hook	Fine Science Tools	10064-14
Vetbond Suture Glue	3M	1469SB
Lipopolysaccharide	Sigma Life Science	L4391
12x12 inch opaque box	Acrylic Display Manufacturing: A division of Piasa Plastics	C4022
Camera/camcorder	JVC	GC-PX100BUS
Covidien Tendersorb™ Underpads	Kendall Healthcare Products Co	7174
WypAll L40	Kimberly-Clark Professional	5600
Surface at 45 degree incline		
Thin wire from a pipe cleaner	Creatology	M10314420
50mL conical tube	Falcon	352070
Fiberglass Screen Wire	New York Wire www.lowes.com	14436
Razor blade	Fisherbrand	12-640
OPTIX 24-in x 4-ft x 0.22-in Clear Acrylic Sheet to make Clear Acrylic Walkway	PLASKOLITE INC	1AG2196A
Protractor	Westscott	ACM14371

Comments/Description (optional)

C57BL/6NCrl is the exact strain we use
Capacity: 25.03 oz. (740mL); Dia. x H: 4.92 x 2.55 in. (125 x 65mm). However, any small round glass container will work. A 2 cup capacity pyrex food storage bowl with flat bottom will also work and is much cheaper (Pyrex model number: 6017399).
Lead ring for stablizing flasks in a water bath. It is used inside the anesthesia dish.

n-butyl cyanoacrylate adhesive
Lipopolysaccaride from e.coli 0111:B4, gamma irradiated

Colored Acrylic 5-Sided Cube, 3/16" Colored Acrylic, 12"W x 12"D x 12"H; http://www.acrylicdisplaymfg.com/htm
Any camcorder that works well in low light and can be imported and edited. We use the JVC GC-PX100 Full HD Everio Camcorder.

Any surface with moderate grip will do
We use a cardboard box.
Any pipe cleaner from any craft store will work.

Any supplier can be used as long as their screen is 16x16 or 18x16
A wooden stick applicator or wooden part of a cotton-tipped swab will also work.
Clear acrylic (1/8" thick) with sides and a top to limit exploration. We bought a sheet of acrylic from a local hardware store and had them cut it to size. (2) 2"x2"; (3) 2"x 18"; (1) 2"x15.5"; (1) 2"x3". Using clear tape, tape all sides together, with the 15.5" piece on top. Tape the 3" piece to the end of the 15.5" piece to create a flap/entryway for the mice. Alternatively, part or all of the walkway can be glued together, and only taping on the top pieces. This design will allow for the walkway to be opened for easy cleaning.

ıl/cubes_19.html

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A battery of motor tests in a neonatal mouse model of cerebral palsy

Author(s): Feather-Schussler DN and Ferguson TS

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Tanya S. Ferguson, Ph.D.
Department:	Shriners Hospitals Pediatric Research Center
Institution:	Temple University School of Medicine
Article Title:	A battery of motor tests in a neonatal mouse model of cerebral palsy
Signature:	Tanya S. Ferguson
Date:	Apr. 10, 2015

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Rebuttal Comments
Click here to download Rebuttal Comments: Response to Reviewers, final.docx

Editorial comments:

Editor's Note: Please note that multiple reviewer # 1 has raised some concerns about aspects of your manuscript. Please thoroughly address or rebut each individual comment below to further strengthen and clarify your submission.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Feather-Schussler and Ferguson presents a battery of functional tests adapted to neonatal mice, and their application to the evaluation of deficits in a neonate mouse model of cerebral palsy.

This summary is accurate, however, misses some key details. This battery of tests was not '*adapted*' for neonatal mice, but was compiled from already published neonatal behavior tests, with appropriate references cited within the manuscript. This manuscript is not claiming to have invented these tests (with the exception of the foot-angle hindlimb ambulation test), but was designed to demonstrate the proper technique for performing a battery of neonatal motor tests using our qualitative scoring sheet, and using JoVE as a platform to visually demonstrate how each of these tests are to be performed. One of the major problems with behavioral tests, especially in neonatal rodents, is that the text descriptions on how to perform these tests and score them are not clear. The current literature does not have good supportive figures or finely detailed descriptions, causing each research group to "modify" the tests and their reporting method. By publishing in JoVE, the video format will show researchers how to perform these tests and report their results using our quantitative scoring sheet so that data can be compared between research groups reliably and reproducibly.

Each test was specifically selected based upon the fact that neonatal mice are able to perform the behavior with reliable and repeatable results. These specific motor tests were chosen to emphasize motor deficits and place less emphasis on sensory-based behaviors. Furthermore, these tests can be used on a wide variety of injury paradigms, whether in our model of cerebral palsy, used as an example model here, or in other neonatal neuromuscular or muscular disorders where motor function is affected.

Major Concerns:

The first part of the paper describes a battery of nine tests based on a subset of tests previously developed by Fox and widely used in a number of studies. The tests are presented with too much approximate age delimitations to be applicable in developmental studies. Thorough analyses of neuro-behavioral developmental testing were already published and are more interesting for developmental

studies (see Leroy et al 2001 for instance).

All reported developmental ranges were taken from previously published papers and were cited, as appropriate and are also listed in the Le Roy 2001 paper, as mentioned by the reviewer (age range from 0-17 days). The tests are to determine whether a motor deficit persists in a neonatal injury model, either over time or following therapeutic intervention (not shown). Additionally, a comprehensive battery of neonatal motor tests is not found elsewhere, including the Le Roy 2001 paper, which is a statistical analysis of Fox's battery of tests with no description regarding scoring or how to perform each test. Our paper highlights motor functions that may be impaired after injury, rather than changes during normal development. Fox (1965) and Wahlsten (1974) test for a combination of behaviors, including the development of motor and sensory functions. Their tests compare the onset of a behavior in the neonate to a mature or adult phenotype with a 0 or 1 scoring system (present or not present). Our paper compares neonatal behavior within the context of an injury paradigm with a more sensitive scoring system and a visual description of how to perform each test. Thus, our paper is unique in this field.

The authors propose to use parametric tests (Student t-test) to analyze the data. This is a misleading procedures in some cases, because several tests use qualitative scoring (ambulation, HL suspension, grasping reflex). Student t-test should not be used with categorical variables: use Chi2, or non-parametric tests at least.

Non-parametric tests are used when data is nominal (yes/no) or ordinal (ranking). These data are interpreted using subjective findings. Non-parametric tests are incorrect and less powerful than parametric tests for our studies. Because the three tests listed above are scored using interval (scale of 1-5 with equidistant points) or ratio (having a true zero point) scales, parametric tests are appropriate; although non-parametric tests can be used with interval or ratio data. However, this statistical analysis would be less powerful. With that being said, a non-parametric Mann-Whitney test for ambulation, hindlimb suspension, and right paw preference in the grasping reflex was determined for comparison sake. The non-parametric p values were extremely similar to the parametric p values and all values were still significant. Additionally, a statistician reviewed the data prior to paper submission and found that all statistical analyses were appropriate. The paper will remain as is.

The second part reports representative results based on the functional evaluation of a mouse model of cerebral palsy carried out on a single measure at P8. The results are limited to differences in the hindlimbs (HIL angles during crawling, HIL Strength and grasping) without allowing to decide if they results from leg weakness or a delay of maturation.

We did perform all tests at a variety of time-points, ranging from 24 hours after surgery (P7) to 1 week following surgery (P13). We reported results 48 hours after surgery (P8), as this time point showed the largest deficits, as part of our “representative results”. Our primary goal was to show there are motor deficits in our model, not the developmental time course of these deficits, nor whether they are due to delayed maturation versus weakness. We are in the process of writing up our full data, including therapeutic intervention, but that was not the purpose of this particular manuscript. As previously mentioned, the purpose of this manuscript was to properly demonstrate how to perform neonatal motor tests complied from a variety of cited sources using the JoVE video format to assure reproducibility and accuracy, using our quantitative scoring methods rather than subjective reporting methods.

A single stage measure does not allow to detect correctly a developmental delay. Neurobehavioral developmental analyses need a longitudinal analysis of the appearance of critical functions. In addition several of these results are based on inappropriate statistical testing.

While the reviewer is correct in that a longitudinal analysis would have to be performed to detect a developmental delay, this was not the purpose of our study. Our intentions were to determine the presence or absence of motor impairment in our cerebral palsy model, which we feel was accurately shown. Additionally, the statistical analysis has been addressed above.

Minor Concerns:
Minor points:
In the Statistical significance chapter the authors suggest to "express data as mean +- SEM". They did not applied it to their own data that are apparently expressed as mean +- S.D. (or the tests are not

Although we cannot assume what the reviewer is attempting to say with the rest of this statement as it is not provided, we can guarantee that the data shown is expressed +/- SEM.

3.2.1. HL foot angle:The measures on the feet should be done better by filming from below on a transparent surface.

We agree with this statement and we have changed the wording to indicate foot angle can be viewed from above or below, as the figure showed. We thank the reviewer for catching this wording omission.

3.8.3 the scoring is not clearly stated.

We have re-worded the grasping scoring and paw preference.

3.7 four limb grip strength. The description of the test is confusing.

We appreciate this comment, but since no specific points of confusion were mentioned, we are unsure what needs editing.

Li323. P8+7days, is probably to late. So you cannot conclude to a delay in the development. It would have been more pertinent do determine the age of acquisition of quadruped locomotion.

We are unclear to what this statement refers. Lines 322-323 are as follows, “2.9.3) Once both the snout and paws have been removed from the edge, stop the timer and record time.” Unfortunately, even trying to determine where this comment came from in the text was impossible, unlike the comments below. If the reviewer would care to clarify, we can appropriately address this comment.

Li328: values for hil and sham are inverted.

Thank you for finding this flaw (line 349-350 in our version)

Li330. This remark is an over-interpretation.

Line 330 does not make any assumption; it is in the methods section. However, we believe the reviewer is concerned about our interpretation of what a wide-based gait indicates. From many human studies, as well as various rodent injury studies, a wide-stance during gait activities is a sign of imbalance or ataxia. We do not feel this remark is an over-interpretation.

Li 345 what is the logic to test at P8 a function that develop after P10 ?

We tested forelimb suspension from P7 to P13 knowing that the selected time point we used for demonstration (P8) was too young for this test. We have clarified the time points we actually examined at the beginning of our representative results section. We still included this test result due to JoVE’s policy that ALL results, positive or negative, are presented. We do have a clear statement within the paragraph that reads, “This test is better for mice older than 10 days”. If the reviewer feels strongly about this result and if it is okay with JoVE, we will remove this result -or- report a different time-point that is within the appropriate age range.

Li 348: decrease in suspension time (data not shown).

Are you referring to the Front-limb suspension test results? If so, does the reviewer suggest that we add the wording, “decrease in suspension time”? However, for forelimb suspension, there is no significant difference in the suspension time. We have adjusted the manuscript to attempt to clarify this section.

Li 356 : Test values for suspension time ?

We misworded this section and thank the reviewer for the comment. We have fixed this statement.

Li 357 P>0.005 ? (idem li 365, 429, 433, 443,452)

We thank the reviewer for catching this “copy and paste” error! The multiple errors have been corrected in the document.

Reviewer #2:

Manuscript Summary:

In this paper, authors created a neonatal mouse model for cerebral palsy, incorporating the major phenotypes associated with the disorder, including hypoxia, ischemia and inflammation. One of the main neonatal behavioral readouts of cerebral palsy is motor dysfunction, which persists into adulthood. Therefore, in addition to creating a neonatal mouse model for cerebral palsy, authors have also attempted to establish a series of repeatable motor tests, designed specifically to test motor function in neonatal mice up to two weeks of age. The surgical protocol and subsequent techniques used to effectively induce cerebral palsy in the neonatal mouse appear to be appropriate. Although the established model is appropriate to test deficits associated with cerebral palsy, I find a few potential problems with some of the behavioral assays testing motor outcomes of the disorder, as I think a few of the assays may cause stress and anxiety to the neonate, which may potentially interfere with the proper testing and outcome of motor function.

The stated summary is very accurate; however, the behavioral tests are well established and thus, the stress and anxiety inherent in these behavioral tests are minimal and do not interfere with the results. All of these tests are well-established and referenced in this and other papers, with the exception of the gait angle test, which is a novel gait analysis test for neonatal mice.

Major Concerns:

For examples of potential methodological confounds:

a) Authors suggest "gentle prodding by touching the pup's tail" (line 182) be used in order to motivate the pup to walk in the Hindlimb foot angle assay. 1) I question the consistency and repeatability of the interaction with the animal across all cases. 2) Instead of motivation, could the prodding cause stress/anxiety in the neonate leading to more freezing behavior?

Because angles of the hind limbs as well as ambulation scoring are not taken when the mouse first begins walking, when the mouse stops waking, nor when the mouse is turning around while walking, the repeatability is high and interaction between observer and mouse does not pose a problem in obtaining data. Furthermore, gentle prodding of the mouse does not cause freezing behavior, increased anxiety or stress, as has been shown previously. Many papers with mouse behavior cite gentle prodding or pinching of the mouse tail to coax the mouse to continue forward movement, including (at random): a 2011 JoVE paper titled “Assessment of Motor Balance and Coordination in Mice using the Balance Beam” by Luong et al.; a 2001 paper published in human molecular genetics titled “The HD mutation causes progressive lethal neurological disease in mice expressing reduced levels of huntingtin” where

gentle prodding of the tail was used to cause adult mice to walk across a surface so hindlimb gait could be determined; and in 1998, the Journal of Cell Biology published a paper titled “AnkyrinG Is Required for Clustering of Voltage-gated Na Channels at Axon Initial Segments and for Normal Action Potential Firing” where authors prodded the tails of mice to observe gait. This is not an exhaustive list of publications in which prodding of the tail has been used to observe gait, therefore, we do not believe gentle prodding of the tail is negatively or inconsistently affecting the results of gait analysis.

b) Surface Righting (line 193). Authors suggest putting the pups on their backs and holding them down, "in position" for five seconds then releasing the pup and recording the time it takes for the pup to return to prone position. Wouldn't forcefully holding the animal down be a confound in the measurement of time it takes for the animal to change position, as it can induce stress/anxiety?

While your concern is reasonable, again, restraining the mouse is not long enough to cause stress and is gentle enough that the pup lays still in this position. While adult mice undergo the “fight or flight” response when turned over, the pups are too young to undergo this fear response. Righting reflex is a common neonatal motor test and the practice of holding the mice on their back is well-established.

c) Cliff Aversion (line 307). Olfactory cues could serve as potential confounds. How do authors plan on removing olfactory cues from the box? Also, if the cliff aversion is used to test for inherent fear, how's that related to motor impairment or vestibular difficulties. It is not clear how an aversion test is measuring motor control without also measuring fear response (line 410).

While it is true that mice respond to olfactory cues, we remove cues by over-scenting the box with many mice. We neglected to add that to our description and thank the reviewer for noticing this. We have now added the box pre-scenting to the description. Pre-scenting the box makes it so the mice do not follow olfactory cues because there are too many cues to follow. In terms of the fear response, the test is examining whether the mice recognize they are at an edge, and then we observe their motor response, which include a horizontal vestibular movement. Motor impairment (not moving or moving with very poor limb coordination) or side preference (turning) is determined when the mouse moves (either to the right or the left) from the edge of the box. The response to turn from the edge of the box is inherent in mice and this test is well established.

Minor Concerns:
Line 78: Inflammation of what region of the brain?

Chronic inflammation is from an unknown source, which may include chorioamnionitis, maternal illness or fetal infection. In this model, inflammation is due to a single injection intraperitoneally of lipopolysaccharide, a well-characterized model of inflammation. As to exactly which areas of the brain react to this global inflammation is unknown, but the animal undergoes a general increase in inflammatory cytokines.

Line 329: this section: 3. Statistical Significance
328 3.1 Using a statistical software analyze the results. Express data as mean ± standard error of the mean (SEM). Tests are parametric so analyzed them by a t-test.

Should be re-written as: (NAME OF SOFTWARE) was used to analyze the results. Data was expressed ad mean +/- standard error of the mean (SEM). Tests were parametric and thus, the data was examined using t-test analyses.

Thank you; we have changed the paper to reflect this suggestion.

Line 371: Authors state that this test is more appropriate for mice older than 10 days, but conduct testing at PND 8. Please provide rationale for this statement.

We tested forelimb suspension from P7 to P13 and have clarified the age ranges tested in our introduction to representative results. We only report one representative time-point (P8) for all tests, knowing that the selected time point was too young for this test. We still included this test in the results as it is a test that could be used for neonatal motor disorders at P10 or later, and JoVE asks for "representative results" for all sections. We can change this result from P8 to another time-point, but it will be different than the other representative time-points reported. We are in the process of publishing a paper with all results, including therapeutic intervention. This paper reports methods of testing neonatal motor deficits using JoVE's video format to demonstrate proper technique using our quantitative scoring sheet to allow for reliable and reproducible testing between research groups.

Additional Comments to Authors:
Overall this is an important and interesting method paper that highlights fairly novel ways to test very young mice for motor control.

We thank the reviewer for his/her kind words.

Reviewer #3:
Manuscript Summary:
This manuscripts describes a battery of tests to detect some forms of cerebral palsy in newborn mice, illustrated by the authors' own results employing the Rice-Vanucci model of stroke caused by unilateral carotid artery cauterization followed by a period of systemic hypoxia.

Major Concerns:
The major strength of the manuscript is the detailed description of the experiments, so investigators

considering to repeat this type of experiments might find valuable Information.

Minor Concerns:

The introduction could be shortened significantly, while a section on pitfalls might be helpful

Thank you for your review. If the reviewer has specific points or parts of the introduction they feel are superfluous, we will be happy to edit them out.

Pitfalls are inherent when working with animals. Although there is not a 'pitfalls' section in the paper, the major pitfalls for some of the tests have been outlined within each test. For example, we suggest the examiner determine the scoring for pups that fall in negative geotaxis, that there can be learning in the absence of negative reinforcement for falling in the hindlimb and front limb suspension tests, and that grasping reflex must be performed prior to 15 days, at which time this reflex disappears (Crawley, 2007: What's wrong with my mouse? Behavioral Phenotyping of Transgenic and Knockout Mice, 2nd Ed).