

Video Article

Assessing Forelimb Function after Unilateral Cervical SCI using Novel Tasks: Limb Step-alternation, Postural Instability and Pasta Handling

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Abstract

Cervical spinal cord injury (cSCI) can cause devastating neurological deficits, including impairment or loss of upper limb and hand function. A majority of the spinal cord injuries in humans occur at the cervical levels. Therefore, developing cervical injury models and developing relevant and sensitive behavioral tests is of great importance. Here we describe the use of a newly developed forelimb step-alternation test after cervical spinal cord injury in rats. In addition, we describe two behavioral tests that have not been used after spinal cord injury: a postural instability test (PIT), and a pasta-handling test. All three behavioral tests are highly sensitive to injury and are easy to use. Therefore, we feel that these behavioral tests can be instrumental in investigating therapeutic strategies after cSCI.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50955/>

Introduction

Cervical spinal cord injury (cSCI) is the most prevalent form of SCI in patients, representing about 62% of all SCIs (<http://www.spinalcord.uab.edu>). Injuries to the cervical spinal cord can result in impairments in the upper extremities as well as breathing. A recent study in patients with cSCI suggests that partial or full function of the arm and/or hand is considered to be a major priority¹. Therefore, developing cSCI models and associated behavioral assays that are easy, sensitive and reliable to use is an important goal. There are several useful behavioral tests to assess forelimb function after cSCI in rodents¹⁻¹⁷, however, many of these tests are difficult to use and require specialized equipment. Pasta handling tests have been developed by other groups^{18,19}, but these tests require that the animals be filmed in specific pasta eating cages, which requires a long training period, while the method we propose can be performed in the animals' home cage. Previously described pasta tests require significant analysis time and often expensive software, unlike the test described here. The goal of our method is to develop and use behavioral tests that are easy to use, reliable, and do not require expensive equipment. Specifically, the forelimb step-alternation test described here allows researchers to estimate lesion volume early after injury, providing a means to create equivalent treatment groups before a chronic treatment is administered. In this study, we describe in detail three novel behavioral tests: (1) forelimb step-alternation, (2) postural instability, and (3) pasta handling.

Determining the right behavioral tests to use can be difficult. Indeed, we feel that no one behavioral test can assess the functioning of the forelimbs adequately. Therefore, we suggest using a combination of a few behavioral tests to assess forelimb function after SCI. Categorically speaking, one should use an open-field locomotor type task (e.g. forelimb locomotor scale, cylinder) where animals are assessed on the use of their forelimb during normal locomotion, and a task specific test, where animals are asked to perform a specific task involving the use of their forelimb (e.g., pasta eating, postural instability, grip strength, etc.). The behavioral tests described here in detail are useful for determining lesion severity at ~ one week post lesion (forelimb step-alternation, postural instability, pasta handling), the effects of a unilateral lesion on the contralesional forelimb (forelimb step-alternation, postural instability) and fine motor movement of the forelimbs (pasta handling). Knowing the severity and effects of the lesion used can also dictate the appropriate behavioral tests. If fine motor movements of the wrist are not possible after lesion, using a test that measures and quantifies the use of forelimb paws is inappropriate. For example, many of the previously described pasta handling tests look more in depth at the individual movements of digits, however, we found with the severity and location of our injury, this was irrelevant and instead observed differences in the overall placement of the limb.

Researchers may be tempted to conduct many behavioral tests and use a sample of them for demonstration based on the results, however, it is recommended that for each experimental model being used a pilot study is performed to determine appropriate behavioral tests for the lesion. Therefore, it is important to note that the user should have at least some prior knowledge of the type of deficit and extent of a particular lesion

type before deciding on behavioral tests to be employed. Assessing lesion severity based on behavioral performance also allows treatment groups to be created with an equal distribution of lesion severity before treatment is administered.

Protocol

All animal procedures were done in accordance with approved protocols with the internal IACUC at the University of Texas at Austin and under National Institutes of Health (NIH) guidelines.

Animals used in this study had a C3/C4 lateral contusion injury using an Infinite Horizon Impactor, or a C3/C4 lateral hemisection model and these methods can be applied more broadly to other models¹⁸. We recommend that researchers test animals for 2 weeks prior to injury, 3 days post injury and regularly (e.g. weekly) for the duration of the study. When performing behavioral tests on post surgical animals, especially at three days post surgery, care must be taken that the animals have recovered sufficiently from the surgery and that the behavioral testing procedures do not cause additional pain or stress to the animals.

In general, 2-3 weeks of handling and pre-testing may be needed prior to surgery. This involves getting the animal comfortable to the testing environment by handling them in a similar way to how they will be handled during testing as well as allowing the animal to move around freely on the testing surface. It is also important to get the animal used to being held, this can be done by moving the animal on and near the testing surface in non-direct motions to acclimate the animal to handling. It is also important to relax the animals at the beginning and in between test sessions. To relax the animals, hold the animal on a tabletop in a forelimb-only bearing stance, in a "wheelbarrow" position and bounce the animal gently on the tabletop allowing the forelimbs to touch the surface. This teaches the animals to sense below them for a stable surface and is an important and necessary step in the procedure because it ensures that the animals are relaxed before testing begins.

1. Forelimb Step-alternation Test

- Test Summary:** Place the animals with both forelimbs touching the tabletop to determine whether they alternate the use of their forelimbs. Move the rat forward, shifting their center of gravity, forcing them to try to step. This test is repeated four times with each animal and if an animal shows ability to alternate at, or higher than, 75%, it is considered to be an alternator. This is a forced motion task, so animals that are considered non-alternators will repeatedly step with the same forelimb.
- Hold an animal on a tabletop in a forelimb-only weight-bearing stance, in a "wheelbarrow" position with their body at nearly 90° from the table.
- Once the animal appears relaxed, push the animal forward to move along the surface of the tabletop.
- Determine and record whether the rat alternates use of the forelimbs while moving across the surface.
- Note and record the forelimb that initiated the movement and whether the animal can alternate or not on the Forelimb Step-Alternation Test Score Sheet (provided).
- Repeat this test at least twice while holding the animal with one hand (right or left hand). Repeat at least twice again while holding the animal in the other hand.
- If rats showed this ability, step-alternation can be tested again by introducing a 5 sec wait after the first step to determine if the pattern of alternation remains after a delay. Note the results and record in the score sheet.
- This test can be used daily, and it is recommended that the animals be tested at least weekly for a repeated measures experiment.

Note: It is important to alternate the experimenter's hand position since it may affect the alternation behavior of the test animals. The Step-alternation score sheet provided can be used to note paw position and use during stepping. Although we have not incorporated those data into our scoring system currently (we have only used alternation status to group the lesioned animals in the current study), users can readily include paw position data as part of the analysis depending on the specific project.

2. Postural Instability Test

- Test Summary:** For this behavioral test, hold rats in a similar position as for the forelimb alternation test, however, each forelimb should be tested individually. The tabletop should be covered with sandpaper (No. 220) to prevent slipping, bracing, or dragging of the forelimbs during the test.
 - In this test, the distance it takes for the animal to take a step with the forelimb being examined to regain its balance will be recorded.
 - Animals within a study group should be approximately the same weight, size and age as large differences in body weight can result in variation in the baseline distance needed for maintaining center of gravity for a given animal¹⁹. A sham group should be included while using PIT to verify that changes in behavior are due to regeneration or recovery rather than age and weight gain.
- Hold an animal on a tabletop in a comfortable stance and allow both forelimbs to reach the surface, the animal should comfortably be in a "wheelbarrow" position with their body at nearly 90° from the table. Note that it is important to hold the animal in a more vertical position. This will allow a more consistent distance needed to trigger a step to regain center of gravity.
- Lightly restrain one forelimb against the animal's torso and align the rat's nose with the zero line as viewed from above.
- Move the rat forward. This will shift the animal's center of gravity forward stimulating the animal to step to regain its balance.
- Record the new position of the nose after the rat steps twice and use the average of these two steps as the distance needed to trigger a step.
- Test each forelimb independently 5x, bringing the animal back to the 0 position and verifying that the animal continues to be relaxed in the experimenter's hands before continuing to ensure consistent results.
- Record on the Postural Instability Test (PIT) Score Sheet, provided. This test can be performed daily, and it is recommended that the animals are tested at least weekly for the duration of the experiment.

3. Pasta Handling Test

1. **Test Summary:** Use this test to determine the time it takes to eat a piece of pasta and the paw preference during a pasta-eating session. In this test, use 4.0 cm strands of dry pasta (thin spaghetti; diameter ~1.6 mm). The test should be administered at approximately the same time each testing day. Rodents generally eat dry pasta readily; however, 4-6 hours of food withdrawal is recommended before testing if this does not occur.
2. Baseline measurements should be acquired 1-2 weeks prior to injury. Prior to baseline recordings, rats should be given the same type of pasta in their home cage to prepare them for testing. Then, allow the rats to eat pasta in a testing chamber and record the time to eat pasta. Pasta pieces should be placed near the front of the testing chamber where the use of forelimbs can be observed readily. Each pasta-eating session should include eating at least three pieces of pasta. Rats are considered proficient in eating pasta if their pasta-eating time is consistent for at least 3 days.
3. After training the rats to eating pasta consistently in the testing chamber, place rats in recording chambers.
4. Place pasta pieces onto the floor near the front of the testing chamber and record time to eat a piece of pasta, forelimbs used, and the positions of the forelimbs, as seen on the Pasta Handling Score sheet.
5. This test can be performed daily, but it is recommended to perform it weekly while observing the animals' weight as to not over-feed the animals.

Note: The Pasta Handling Score sheet provides space to record time to eat pasta and paw use, similar to the data included in this study. We have also included areas to record additional details of paw use during a pasta eating session, such as paw position.

Representative Results

We recommend that animals be tested regularly throughout the experiment in a repeated measures-type experiment for improved analysis and to analyze improvement over time.

Forelimb Step-alternation Test

This test is designed to determine an animal's ability to alternate the use of its forelimbs. Using the forelimb step-alternation test, only 50% of the animals were able to alternate the use of their forelimbs after cSCI¹⁹ at 12-16 weeks post injury (**Figure 1A**). Next we introduced a delay (5 sec) by holding the animal stationary after the first step before moving the animal forward for the second step. Only 50% of the animals that were alternating the use of their limbs (25% of the total lesioned group) were able to alternate after a 5-sec delay (**Figure 1B**). We correlated these findings with anatomical studies and the analysis revealed that alternators have significantly larger area of spared corticospinal tract [$F(1,1) = 5.56, p < 0.05$] and dorsal column [$F(1,1) = 19.2, p < 0.003$] on the contralesional side¹⁹. These data indicate that the forelimb step alternation test can predict the severity of the lesion as well as unilaterality of the lesions. This test can result in nominal categorical data that should be analyzed with a repeated measures analysis. In this example, it was not a repeated measure, we used one time-point to illustrate the difference between groups. Each animal will be categorized as an "alternator" or a "non-alternator", separating the animals into two groups of injuries (moderate vs. severe, respectively).

Postural Instability Test

Unilateral lesions of the spinal cord can cause changes not only to the impaired and/or affected limb, but also to the non-impaired limb. The postural instability test (PIT) was previously described and used in animals with a rodent model of Parkinson's disease¹⁸ and in a rodent model of cSCI¹⁹. We used PIT after cSCI and found that there was a significant effect of lesion status [$F(1,1) = 8.17, p < 0.01$]. In addition, forelimb displacement (distance to regain center of gravity) was significantly larger in the ipsilesional side (right forelimb) of injured animals compared to animals with sham surgery (6.00 ± 0.24 cm versus 8.00 ± 0.10 cm, $p < 0.0001$; **Figure 2**). The displacement distance on the contralesional forelimb was significantly smaller in lesioned animals compared to sham-operated animals (6.00 ± 0.25 cm for sham versus 4.00 ± 0.10 cm for cSCI, $p < 0.0001$; **Figure 2**). This result indicates that contralesional forelimb also resulted in changes due to lesion to the opposite side (*i.e.* changes in postural adjustment by the contralesional forelimb to regain center of gravity). The data from this test will be quantitative and should be analyzed using a repeated measures ANOVA to examine changes in groups throughout an experiment.

Pasta Handling Test

Pasta handling was designed to test the skilled use of the forelimbs while eating a piece of dry pasta. A similar test has been used previously to detect deficits in skilled use of forelimbs in animal models of unilateral stroke and Parkinson's disease^{20,21} and after cSCI¹⁹. At chronic time points after injury (12 weeks post-injury), all sham animals ate pasta using both paws during the test period. In the lesioned group, we found that overall time to eat a piece of pasta is similar to the control group (**Figure 3**). However, more alternators (milder injury; 10 out of 15) were able to use their ipsilesional forelimb compared to non-alternators (severely injured group; 1 out of 7; **Figure 3**). The pasta handling test provides data that is both continuous and quantitative (time to eat pasta), and categorical (paw use) and should be analyzed appropriately.

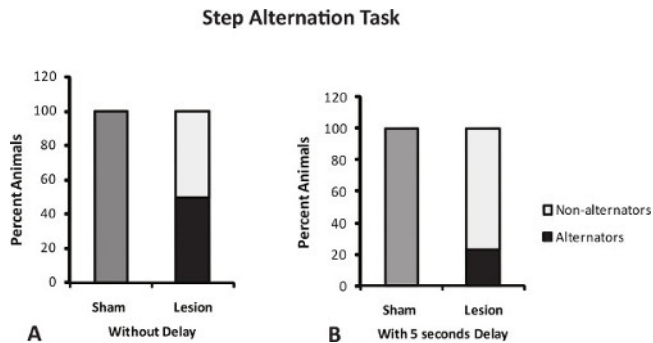


Figure 1. Chronic assessment of forelimb function using the forelimb step-alternation test revealed two different groups of animals with different lesion severity. Animals showed a difference in behavior during forward stepping using both paws. Only 50% of the lesioned animals alternated paws while stepping (alternators; 5 out of 10 lesioned rats), whereas the others showed a lack of step-alternation by taking two contralesional steps in a row (non-alternators; 5 out of 10 lesioned rats). More animals displayed this tendency to take multiple contralesional steps if a 5-sec delay was introduced between steps (8 out of 10 lesioned rats).

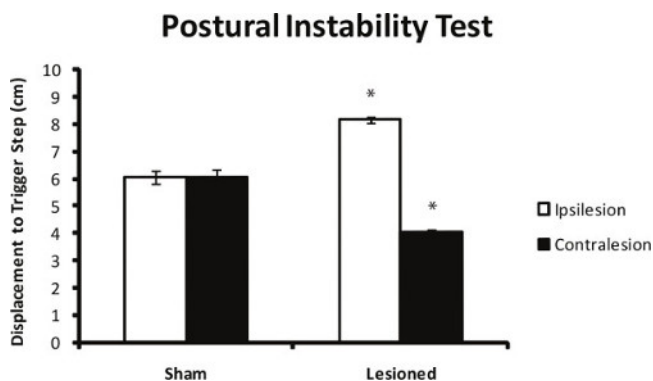


Figure 2. Chronic forelimb behavioral assessment using the postural instability test (PIT). As expected, the ipsilesional forelimb in animals with cervical spinal cord injury (cSCI) had significantly larger displacement distance than animals with sham surgery (6 cm versus 8 cm; $*p < 0.0001$). In addition, the displacement distance on the contralesional forelimb was significantly smaller in lesioned animals compared to sham operated animals (6 cm versus 4 cm; $*p < 0.0001$). This may suggest enhanced function of the contralesional limb during PIT in lesioned animals.

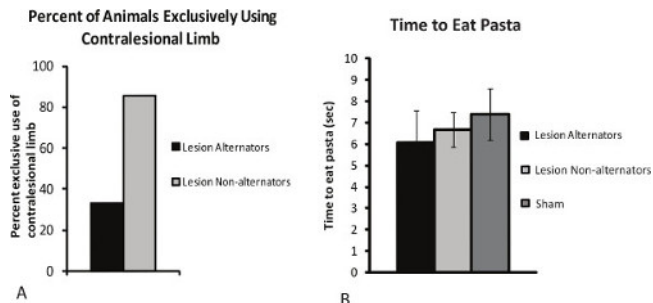


Figure 3. Forelimb assessment using the pasta handling and eating test showed significant impairments in lesioned non-alternators. The animals were given a standard piece of pasta. Paw use and time to eat pasta were recorded. The results indicated that a low number of alternators (33%) exclusively used their contralesional forelimb (indicating more severe impairment) compared to non-alternators (86%). In addition, animals in all three groups (sham, lesioned alternators, and lesioned non-alternators) took a similar length of time to eat the pasta ($p < 0.05$). [Click here to view larger figure.](#)

Discussion

Cervical spinal cord injury (cSCI) can result in devastating and life altering injuries in patients. There are a number of cervical spinal cord injury models developed in rodents that are used to study plasticity of the neural substrates and therapeutic agents. Developing sensitive, effective, reproducible and easy to use behavioral tests to assess functional deficits and recovery after cSCI is an important goal. Here, we describe in detail the use of three such behavioral tests: limb alternation, postural instability and pasta handling.

Spinal cord injury in rodents, as in the human population, is a heterogeneous injury. A very similar injury can induce a variety of behavioral deficits. Therefore, it is important to determine the severity of the lesions in injured animals. We found that the limb alternation task is a very effective way of determining if the contralesional side is damaged after a unilateral injury (hemisection or contusion type injury) to the cervical spinal cord¹⁹. Data from our group showed that the use of this limb alternation task can determine the severity of the injury within the first week after injury. In addition, the more severely injured animals (non-alternators) had a significantly different recovery profile compared to alternators

(using the forelimb locomotor scale). Therefore, the limb alternation task is extremely useful in grouping the injured rats into alternators and non-alternators, particularly if the study includes chronic treatment to ensure there is an equal distribution of lesion severity in each treatment group.

It is recommended that the forelimb-alternation test be used in combination with other tasks that can examine the use of animals' forelimbs (such as the forelimb locomotor scale or the cylinder paw preference test). While administering this test it is imperative that the experimenter perform the test the same number of times with each hand (e.g. twice with the left hand and twice with the right) to minimize the effect of experimenter hand position on the rats' behavior. This test is only effective if the animal is relaxed in the experimenter's hands; the 2-3 weeks of handling suggested should afford relaxation. Further, once the animal is relaxed, the experimenter should hold the animal at nearly 90° in a wheelbarrow position to prevent within-animal variation.

The forelimb-alternation test can also provide insight to cross-spinal neural connections when animals are unable to step individually with a paw but are able to perform an alternation task. For normal quadrupedal locomotion in animals such as rats, many brain regions as well as local central pattern generators (CPGs) along the length of the spinal cord are involved. Specifically for forelimb rhythmic movement, the cervical spinal segments, C3-C6 are thought to be important²². Previously, we described anatomical differences between alternators and non-alternators in lesioned animals (a lateral hemisection at C3/C4 was performed) and found more damage to the contralesional dorsal column and corticospinal tract in non-alternating animals¹⁸. It is thought that for forelimb movement, rhythmic pattern generator is present at each level of the cervical hemi-segment and that the cross-cord inhibitor connections are responsible for alternation²³. Our previous anatomical findings also implies that damage to the contralesional side of the spinal cord may cause disruption to the rhythmic motor output (such as alternation), whereas if the contralesional of the spinal cord is more intact, the rhythmic motor outputs are more likely to function normally. We did not observe significant damage to any contralesional medial ventral portions of the cords and hence that analysis was not performed.

The postural instability test described here is a very useful test because it can detect changes in both forelimb (ipsi- and contralesional) caused by a unilateral lesion. It is important to note that this test should be administered only when the animal is relaxed in the experimenter's hands. In our experience, this can take daily handling of the animals for 2-3 weeks. Once the experimenter and the animal are comfortable, this test is very sensitive and can be used to obtain highly consistent displacement data for each forelimb. If the animal is tense, the experimenter can gently hold the animal and move them up off of and down onto the table until the animal is comfortable and understands that the table is a safe location. Once the animal is relaxed, it is important to hold the animals in a wheelbarrow position at nearly 90° for consistent results. This test is easy to administer and provides insight beyond other behavioral tests by providing information about compensation and deficit with one simple task.

Rats readily eat dry pasta after training in front of the camera. Therefore, pasta handling is a relatively easy test to administer. In this study, we were recording and using data for limb use (left, right or both) and time to eat pasta only. A more detailed analysis including paw adjustments during pasta eating has been described previously in animals with injuries such as unilateral ischemic lesions and unilateral striatal dopamine depletion^{20,21,24}. Therefore, it is possible to develop and use a more sensitive analysis from the pasta eating test after cSCI. We included paw position on the scoring sheet, as this may reveal handedness or ability with each hand. Further, we are noting the grip of each paw; some animals rest their paw on one side of the pasta, using it possibly as a support, rather than grip the pasta during eating. If animals are hesitant to eat pasta, a food deprivation step can be introduced to the protocol prior to testing.

Disclosures

No conflicts of interest declared.

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