Video Article

Acute and Chronic Tactile Sensory Testing after Spinal Cord Injury in Rats

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URL: http://www.jove.com/video/3247/
DOI: 10.3791/3247

Keywords: Medicine, Issue 62, Rat, neuropathic pain, allodynia, tactile sensation, spinal cord injury, SCI, von Frey monofilaments,

Date Published: 4/4/2012

Citation: Detloff, M.R., Fisher, L.C., Deibert, R.J., Basso, D.M. Acute and Chronic Tactile Sensory Testing after Spinal Cord Injury in Rats. J. Vis. Exp. (62), e3247, DOI : 10.3791/3247 (2012).

Abstract

Spinal cord injury (SCI) impairs sensory systems causing allodynia[1-8]. To identify cellular and molecular causes of allodynia, sensitive and valid sensory testing in rat SCI models is needed. However, until recently, no single testing approach had been validated for SCI so that standardized methods have not been implemented across labs. Additionally, available testing methods could not be implemented acutely or when severe motor impairments existed, preventing studies of the development of SCI-induced allodynia[3]. Here we present two validated sensory testing methods using von Frey Hair (VFH) monofilaments which quantify changes in tactile sensory thresholds after SCI[4-5]. One test is the well-established Up-Down test which demonstrates high sensitivity and specificity across different SCI severities when tested chronically[5]. The other test is a newly-developed dorsal VFH test that can be applied acutely after SCI when allodynia develops, prior to motor recovery[4-5]. Each VFH monofilament applies a calibrated force when touched to the skin of the hind paw until it bends. In the up-down method, alternating VFHs of higher or lower forces are used on the plantar L5 dermatome to delineate flexor withdrawal thresholds. Successively higher forces are applied until withdrawal occurs then lower force VFHs are used until withdrawal ceases. The tactile threshold reflects the force required to elicit withdrawal in 50% of the stimuli. For the new test, each VFH is applied to the dorsal L5 dermatome of the paw while the rat is supported by the examiner. The VFH stimulation occurs in ascending order of force until at least 2 of 3 applications at a given force produces paw withdrawal. Tactile sensory threshold is the lowest force to elicit withdrawal 66% of the time. Acclimation, testing and scoring procedures are described. Aberrant trials that require a retest and typical trials are defined. Animal use was approved by Ohio State University Animal Care and Use Committee.

Video Link

The video component of this article can be found at http://www.jove.com/video/3247/

Protocol

1. Up/Down Plantar Von Frey Testing

The purpose of the test is to determine tactile sensitivity by measuring how much force is required to elicit paw withdrawal from the stimulus. Commercially available calibrated Von Frey Hair monofilaments between the ranges of 4.56 and 6.10 are used to stimulate the paw. Other sensory input like visualization of the stimulus or proprioception can confound tactile threshold measures so responses based on visual or proprioceptive input are invalid. To prevent visualization of the monofilament touching the skin, we test only while the rat is eating cereal. This cognitive distracter, also ensures that all rats are tested at similar baseline levels of comfort. To avoid responses due to proprioceptive rather than tactile input, we do not include trials where the monofilament lifts or moves the paw.

A. ACCLIMATION PROCEDURES FOR THE UP-DOWN APPROACH

1. Present fruit cereal in the home cage so rats recognize and seek the food reward. Rats do not need to be food deprived at any point in the gentling or testing process.
2. Rats are ready to be acclimated to the testing environment and procedures once they eat fruit cereal in the home cage. Acclimation occurs over at least 4 sessions of 10 minutes each but more may be needed. Ten minute sessions are used so that the rats do not become satiated before the end of the session.
3. Place the rat in the testing chamber with three pieces of fruit cereal. Provide additional fruit cereal one to two at a time as they are eaten. On the third session while the rat is eating, produce hand movements under the wire floor so that the rat becomes accustomed to movement below its paw. On the fourth session, apply the 5.18 monofilament to the plantar surface of the paw approximately once a minute for a total of ten presentations. A naïve rat should not respond to this sub threshold stimulus.
4. Tally the pieces of cereal consumed, frequency of urination and defecation, freezing, and grooming behaviors. Freezing behavior occurs in two ways: a) the rat is crouched low to the ground usually near the back wall or facing away from the examiner and remains motionless; b) the rat is hunched over in the middle of the cage and usually chatters its teeth. Freezing behaviors may continue for long periods of time.
5. A gentled rat that is ready for testing will eat nearly continuously for 10 minutes. It will move easily in the cage and seek fruit cereal very quickly after consuming the last. Importantly, the rat will not stop eating or be distracted by hand movements under the wire floor or monofilament application to the paw.

B. TESTING PROCEDURES FOR THE UP-DOWN APPROACH

1. Rats produce distress vocalizations that are above our human aural range. Rats waiting to be tested should be kept in a separate room so that they can not hear these vocalizations.
2. Rats with spinal cord injury that are appropriate for up-down von Frey Hair testing must demonstrate weight bearing with the limbs. Limbs that are unable to support the rat cannot be reliably tested because the application of monofilaments moves the paw before the hair bends.
3. Place a rat in the testing chamber with 2 or 3 pieces of fruit cereal. Rats must be eating during each VFH application.
4. Roll a die to determine which HL to start testing first. Even numbers represent the right paw and odd numbers represent the left.
5. Begin the testing session with the 5.18 monofilament. Withdrawal responses can occur from 4.56 to 6.10 in normal and SCI rats and the 5.18 monofilament falls in the middle of the range.
6. Do not apply a monofilament while a rat freezes, crouches or does not eat. If the tester waits, sometimes these conditions will resolve and testing can resume. If the rat displays these behaviors and the total time in the apparatus is 20 minutes, stop the testing and retest from the beginning the next day. Do not remove the rat while it is displaying these behaviors as this may increase their occurrence. Wait until the rat breaks out of these patterns before handling it to avoid reinforcing these behaviors.
7. Touch the monofilament to the L5 plantar dermatome of the hind paw between the foot pads until it bends (see **Figure 1A**). This smooth steady application should take about one second then remove it from the skin. Avoid touching any part of the foot pads as the sensitivity of the skin is much lower in these areas. This single touch constitutes one trial.
8. A positive response occurs when the rat quickly lifts the paw up away from the monofilament. The withdrawal must be initiated by the time the monofilament is removed in order to confirm that the response is associated with the monofilament application.
9. Watch the animal for other evidence of awareness of the monofilament touch such as vocalization, looking downward, backward or at the tested paw, licking the paw, sustained paw withdrawal or twitching the ears. Note them on the data sheet. When these behaviors occur with paw withdrawal it serves as evidence of supraspinal perception of the stimulus.
10. If there is a positive response, apply the next lowest monofilament. If there is no response (negative), move up to the next higher monofilament. Allow 30 seconds between each monofilament application for the sensory receptors to come back to baseline firing before retesting (to prevent adaptation).
11. Continue applying higher or lower monofilaments to the hind paw based on positive or negative responses until you have 10 trials. Then repeat on the other hind paw. (see example datasheet).
12. The highest VFH that can be tested without introducing proprioceptive input and lifting the paw is 6.10. The 6.45VFH and larger should not be used because the paw lifts before the monofilament bends. If there is no response at 6.10 then the next trial will apply the 6.10 again.
13. A response should be voided and the trial must be re-tested if one of the following occurs during the stimulus application or paw withdrawal:
    a. The rat is not continuously eating.
    b. The rat moves away from the VFH stimulus prior to the bending of the monofilament, making it impossible to know the force applied that elicits the withdrawal response.
    c. The paw is lifted by the VFH (either before or after the VFH is bent) providing proprioceptive in addition to tactile stimulation.
14. A testing session usually can be completed in less than 15 minutes for both hindlimbs.

C. DATA PROCESSING FOR THE UP-DOWN APPROACH

1. A successful test will have 10 sequential trials for each paw.
2. The value of the tactile threshold is the lowest monofilament that produces paw withdrawal on ≥50% of the presentations and 3 or more presentations of the monofilament have be given (see example datasheet, thresholds are circled).
3. A monofilament with only 2 total trials is not selected as the threshold even when one trial is positive and one is negative. Multiple presentations of the stimulus are better indicators of the pattern of responses to that force.
4. If there is no monofilament that produces positive responses on at least 50% of the tests, the rat is given a score of 6.45. This means that the rat did not detect the highest testable monofilament of 6.10 beyond random chance. A value of 6.45 is given because it represents the next value higher than the testable range of monofilaments.
5. Convert the threshold value for the right and left paws into gram force. The gram force values will be provided by the supplier of the von Frey hairs. . For data from bilateral injuries, the threshold gram force for the two paws can be averaged together to yield a single score.

2. Dorsal Von Frey Testing

The purpose of this test is to determine tactile sensitivity in rats with such poor motor control that they cannot be tested using the typical plantar VFH methods. It quantifies how much touch force is needed to produce an aversive response. To avoid responses to proprioceptive stimuli, we do not include trials in which the paw is moved by the VFH during application. Additionally, we do not include trials in which the VFH slips or is brushed across the dorsal surface of the foot.

A. ACCLIMATION PROCEDURES FOR DORSAL VON FREY TESTING

1. Gentle rats for four sessions of 10 minutes each prior to baseline testing and spinal cord injury.
2. Loosely wrap the rat in a small towel so the hindquarters are exposed as shown below (**Figure 1B**). Position the rat over a table with the hind paws lightly resting on the surface. Use the same snug but not tight wrap and hold for each rat. Bracing your forearm on the table edge may reduce variability between rats.
3. A gentled rat that is ready for testing will not resist being held in the towel for several consecutive minutes; however you may have to re-wrap the rat 1 or 2 times during the testing of both hind paws.

B. TESTING PROCEDURES FOR DORSAL VON FREY TESTING

1. Roll a die to determine which hind limb to start testing first. Even numbers represent the right paw and odd numbers represent the left.
2. Loosely wrap the rat in the towel leaving the hindquarters exposed.
3. Tactile stimulus is applied only after the rat is calm and quiet for at least 10 seconds.
4. Starting with the 4.56 VFH, apply the monofilament perpendicularly to the dorsal surface in between the first and second metatarsal approximately 1 cm proximal to the joint (**Figure 1C**). Apply the monofilament in a smooth, steady manner over a one second interval until it bends then remove it. This single touch constitutes one trial.
5. A positive response occurs when the rat quickly moves the paw away from the stimulus. The direction of motion is often posterior or external before the hind limb is flexed. The withdrawal must be initiated by the time the monofilament is removed in order to confirm that the response is associated with the monofilament application.

6. Apply the 4.56 monofilament twice more with at least 30 seconds between applications in order to prevent sensory "windup" and to allow the sensory receptors to come back to baseline firing before retesting (to prevent adaptation).
7. A stimulus test should be reapplied if the VFH slips and gently grazes the skin, making the force delivery more of a brushing or a sweep rather than a pointed contact.
8. If there are fewer than 2 (i.e. 1 or 0) positive responses to the 4.56 monofilament, the next larger VFH is applied 3 times in the same manner.
9. In this way, progressively larger VFHs from 4.56 through 5.88 are applied until two or more positive responses occur or until the largest VFH is reached.
10. The paw withdrawal threshold is the lowest VFH which elicits at least 2 positive responses out of 3 trials.
11. Repeat on the other paw.
12. A testing session usually can be completed in 15 minutes for both hind paws.

3. Representative Results

A successful test will result in immediate, brisk withdrawal of the hind paw to a perpendicularly applied VFH to the mid-plantar region (**Figure 1A**) or the dorsal surface between the 1st and 2nd metatarsal 1 cm from the joint (**Figure 1C**). These responses are tallied to derive tactile threshold for each hind paw. Tactile thresholds from dorsal VFH tests in acute SCI showed excellent fidelity with plantar VFH thresholds derived at chronic times (**Figure 2**). Declines in tactile thresholds derived from plantar or dorsal tests occur for graded SCI (**Figure 3**[3,5]). This hypersensitivity manifests as early as 7 days after SCI and is maintained for the duration of the experiment[3-4,6]. While a range of SCI severities do not affect tactile sensory thresholds of the paw, declines in thresholds occur for more severe SCI (**Figure 3**). Importantly, the development of below-level allodynia is an all-or none response that occurs when there is a loss of more than 60% of the white matter at the lesion epicenter. (**Figure 4**[3]).

Tactile thresholds can decay and produce artificial responses to low VFH forces. At least two conditions can produce threshold decay - rapidly applying the VFH to the skin in a poking or jabbing manner or not waiting long enough between stimulus applications. To be confident that the tactile thresholds are genuine, care must be taken to apply each VFH using a similar low velocity approach and the interstimulus interval must be at least 30 sec. We have also shown that eating behavior reduces threshold decay[5].

Figure 1. Schematic and photograph depicting the location of VFH stimulation for the plantar and dorsal von Frey hair (VFH) testing. A) For plantar VFH testing, the von Frey hair should be applied perpendicularly to the mid-plantar hindpaw approximately 1 cm posterior to the middle phalange (as indicated by the red box in the diagram of the plantar surface of the hindpaw) at a slow consistent speed until it bends. B and C) For the dorsal VFH technique, the rat is loosely wrapped in a surgical towel as shown in the photograph. The VFH is applied to the dorsal surface of the paw in between the first and second metatarsal approximately 1 cm proximal to the joint.

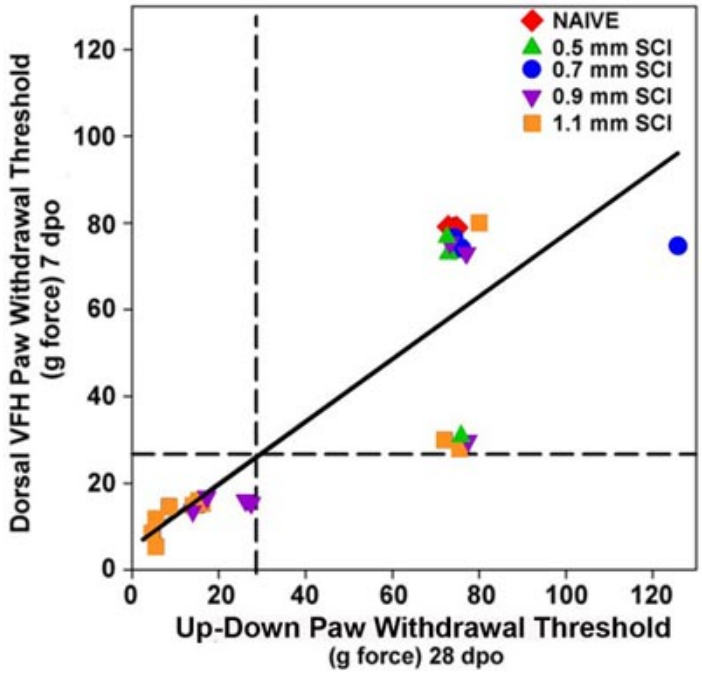

Figure 2. Dorsal von Frey Hair sensory thresholds correlate to standard plantar VFH. (n-44; Pearson Coefficient = 0.503, $p < 0.01$, $r2 = 0.67$). Dashed lines show the cutoff threshold for allodynic sensation using either the Dorsal VFH or the plantar VFH techniques and indicate that allodynic thresholds at 7 days after SCI using the Dorsal VFH test predict chronic below-level allodynia determined by plantar VFH methods.

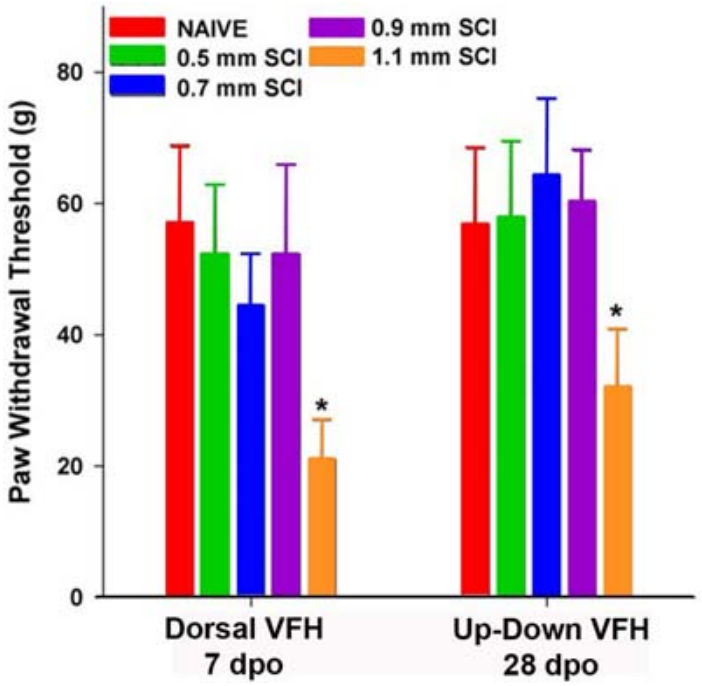

Figure 3. Early dorsal von Frey Hair sensory thresholds predict allodynic and non-allodynic tactile sensitivity across injury severities at later timepoints. Comparable sensory thresholds occurred between 7 days post SCI Dorsal VFH and 28 days post SCI plantar VFH measures. Remarkable similarity occurred for a wide range of SCI, with significant allodynia noted in moderate to severe SCI ($p<.05$ 1.1 mm displacement vs. all other groups).

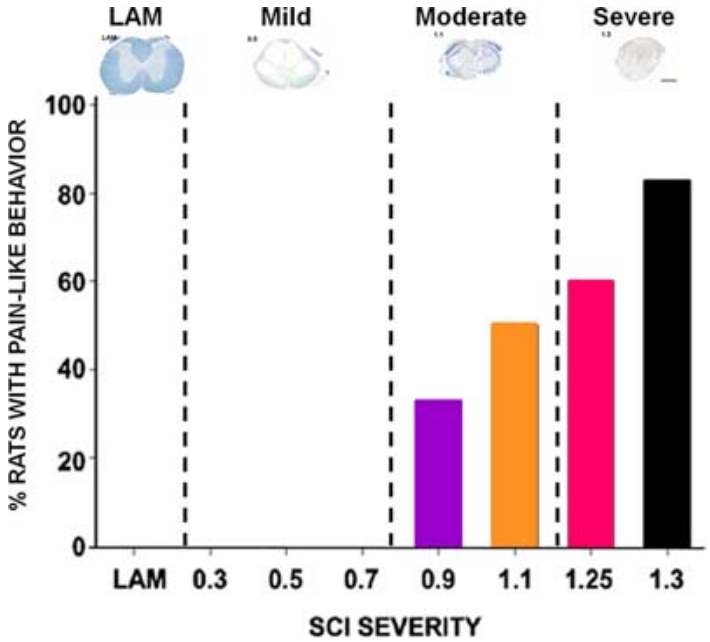

Figure 4. Percentage of rats receiving laminectomy (no SCI) and SCI groups showing hindpaw allodynia at 6 weeks after SCI. Allodynia was only seen in rats with moderate or severe SCI (OSU ESCID device 0.9, 1.1, 1.25, 1.3 mm cord displacements) while rats with mild SCI (0.3, 0.5, 0.7 mm spinal cord displacements) exhibited normal responses to tactile stimulation. Representative coronal spinal cord sections stained for myelin at the lesion epicenter for laminectomy (uninjured control), mild (0.5 mm displacement), moderate (1.1 mm displacement) and severe (1.3 mm displacement) spinal cord contusions are included to show the degree of axonal sparing that is associated with this hypersensitivity. (Scale bar = 0.5 mm).

VON FREY HAIR TESTING

Rat # 106 Date 5/9/10 Study SCI 118

DPO 56 Tester MB Time 1:45-2:01

Trial	Paw	Von Frey Hair	Withdrawal	Comments
1	Right	5.18	--	
2		5.46	+	GL
3		5.18	--	
4		5.46	+	
5		5.18	+	
6		5.07	--	
7		5.18	+	
8		5.07	--	
9		5.18	+	
10		5.07	+	

5.07 + — / ||

5.18 (circled) + — /// ||

5.46 + — ||

Trial	Paw	Von Frey Hair	Withdrawal	Comments
1	Left	5.18	--	
2		5.46	--	
3		5.88	+	
4		5.46	--	
5		5.88	+	LK
6		5.46	+	
7		5.18	--	
8		5.46	+	
9		5.18	+	
10		5.07	--	

5.07 + — /

5.18 + — / ||

5.46 (circled) + — || ||

5.88 + — ||

Supraspinal: GL = glance backwards to tested side, FT=looked at foot which is held off the ground, LK = licked foot, VC = vocalized following stimulus

Figure 5. A sample data sheet for up-down von Frey hair testing. The hair value and the result (positive or negative) are recorded for each trial. Indications of supraspinal awareness of the testing are noted. At the end of the test, the responses at each hair value are tallied. The selected threshold value for the example data is circled.

Discussion

The VFH testing methods presented here represent two testing paradigms that are sensitive and valid for use in rat SCI models. Importantly, the dorsal VFH test was created for assessment of the recovery of tactile sensation of the hind paws acutely following mid-thoracic SCI when rats are unable to stand on their hindquarters. This testing paradigm may also be used when rats regain the ability to support their weight and for the hind limbs after cervical SCI.

The two paradigms demonstrated here utilize calibrated VFH to determine the tactile threshold of each hind paw. Other methods determine the frequency of withdrawal to either a normally innocuous or normally noxious stimulus. We have previously shown that small improvements in tactile thresholds may represent the difference between the presence of chronic neuropathic pain and normal tactile sensation. Hence utilizing experimental testing methods that derive pain thresholds may facilitate translation of potential therapeutic interventions to the clinic.

As with all behavioral testing, it is imperative to minimize the number of confounders within the testing environment that may affect the outcome of the behavioral test[9]. Thus, it is important that each testing session occurs at the same time of day, in the same temperature and humidity controlled room. In addition, since rats communicate vocally in an ultrasonic range, it is important that rats waiting to be tested are kept in a separate room until their testing commences. Langford *et al*[10] showed dramatic evidence that naïve animals experience pain after witnessing a cagemate exposed to noxious stimulation. To further eliminate variability in tactile sensory testing, we utilize sugared cereal as a cognitive distractor during plantar VFH testing. In so doing, we stabilize the responses of the animal. The threshold is less likely to decay throughout testing when a distractor is provided than when one is not[5]. This stabilization is likely due to the tempering of hypervigilant and catastrophizing behaviors, two anticipatory states where the threat of pain activates fear and emotional centers in the brain.

Disclosures

The authors have nothing to disclose.

Acknowledgements

NIH NS43798 (DMB); NIH F31 NS058138 (MRD); P30-NS045758 (CBSCR); and the Paralyzed Veterans of America #2451 (DMB) and #2707 (MRD).

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