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Unilateral T13 and L1 Dorsal Root Avulsion: Methods for a Novel Model of Central Neuropathic Pain

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Abstract

Central neuropathic pain is associated with many disease states including multiple sclerosis, stroke, and spinal cord injury, and is poorly managed. One type of central neuropathic pain that is particularly debilitating and challenging to treat is pain that occurs below the level of injury (below-level pain). The study of central neuropathic pain is commonly performed using animal models of stroke and spinal cord injury. Most of the spinal cord injury models currently being used were originally developed to model the gross physiological impact of clinical spinal cord injury. In contrast, the T13/L1 dorsal root avulsion model of spinal cord injury described here was developed specifically for the study of central pain, and as such, was developed to minimize confounding complications, such as paralysis, urinary tract infections, and autotomy. As such, this model induces robust and reliable hindpaw mechanical allodynia. Two versions of the model are described. The first is optimal for testing systemically administered pharmacological manipulations. The second was developed to accommodate intrathecal application of pharmacological manipulations. This model provides an additional means by which to investigate central pain states associated with spinal cord injury, including below-level pain. Finally, a brief discussion of at-level pain measurement is described as it has been suggested in the literature that the mechanisms underlying below- and at-level pain are different.

Keywords

Spinal cord injury; Hindpaw allodynia; Grid walk; Central neuropathic pain

1. Introduction

Central neuropathic pain is commonly associated with various disease states including stroke, multiple sclerosis, traumatic brain injury, tumors, epilepsy, and spinal cord injury. Chronic pain is common following spinal cord injury, affecting 65% of the patients. Of the patients experiencing chronic pain, 34% described the pain as severe and intolerable (1–3). Extreme pain adversely impacts psychological and social functioning (4, 5), and inhibits rehabilitative efforts (6). While animal models of spinal cord injury are used to study central pain, they were originally developed to mirror the clinical gross pathology associated with traumatic spinal cord injury (7–11). As such, the degree of damage and loss of voluntary motor control associated with these models are not ideal for exploring mechanisms underlying central neuropathic pain.

The avulsion model described here was developed with the expressed purpose of studying central neuropathic pain. Neuronal activity measured in spinal cord injured patients

experiencing neuropathic pain and having avulsed roots shows spontaneous neuro-electrical hyperactivity at the avulsion site (12). Our goal was to cause an avulsion spinal cord injury that induced pain 4–6 dermatomes below the injury site. This was the explicit goal because such a model would allow discrete spinal cord dorsal horn injury (T13/L1) to be located sufficiently rostral to the hindpaw dermatomes (L5/L6) so to avoid any confounding damage to sensory/motor function of the hindlimbs. Consistent with this, avulsion of T13 and L1 induces hindpaw allodynia (Fig. 1). Unilateral avulsion of either T13 or L1 dorsal roots alone does not result in robust and reliable below-level pain, whereas combined unilateral avulsion of T13 and L1 dorsal roots induces robust and reliable hind paw allodynia (Wieseler et al., under review). Hindpaw allodynia develops over time, with reliable allodynia developing (compared to sham-operated controls) by 3 weeks postsurgery and lasting approximately 9 weeks postsurgery. The avulsion causes discrete tissue damage limited to the dorsal horn.

Here, the detailed method for unilateral T13/L1 dorsal root avulsion model is presented. Two versions of the model are presented. The first is a surgical procedure that is shorter in duration and suitable for the study of systemically administered pharmacological agents. The second requires a longer surgical period due to inclusion of suturing the dura. This second method was developed to allow for intrathecal application of pharmacological manipulations to the injury site after re-establishment of the CSF space upon healing of the incised dura. Finally, in order to test motor effects, the method for assessing behavior on the grid walk is described.

2. Materials

2.1. Avulsion Surgery

1. Scalpel handle and No. 10 scalpel blades.
2. Two #5 stainless steel straight fine tip forceps (Fine Science Tools, #11254-20), one pair with the tips bent to form a slight hook.
3. #7 Stainless steel curved fine tip forceps (Fine Science Tools, #11274-20).
4. Fine-tipped Rongeurs (Fine Science Tools, #16021-14).
5. Hemostats (Fine Science Tools, #13004-14).
6. Toothed forceps (Fine Science Tools, #11023-14).
7. Suture hemostats with scissors (Fine Science Tools, #12502-14).
8. 4-mm spring scissors (Fine Science Tools, #15018-10).
9. Small scissors (Fine Science Tools, #14058-11).
10. 23-G $\frac{3}{4}$ in. hypodermic needle.
11. 22-G $\frac{1}{2}$ in. hypodermic needle.
12. 1 cc Syringe.
13. Sterile Q-tips to absorb blood.
14. Sterile gauze (5.08 cm $\times$ 5.08 cm).

15. Magnifying glasses (Cabela's Inc., Sidney, NE; #IJ-711125, 3×).
16. Gelfoam sponge sheets (Upjohn, Kalamazoo, MI).
17. Oxycel (cut into 1 mm × 1 mm pieces stored in a sterile vial until needed; for absorption of blood).
18. 500- μ L Centrifuge tubes.
19. Sterile silk suture (3–0) with attached needle (cutting FS-1; Ethicon, Somerville, NJ).
20. 16 mm Stainless steel Michel clips (Alimed, Dedham, MA).
21. Wound clip applicator (Fine Science Tools, #12028-12).
22. Shaver.
23. Ultra hot glass-bead sterilizer to autoclave surgical tools.
24. Foam padding (~4.31 cm thick, purchased from fabric store).
25. Cardboard boxes (sized to fit within home cage).
26. Draping material (e.g. paper towel with a hole cut in the center to allow access to the surgery site).
27. Sterile silk suture (6–0) with attached needle (cutting P-1; Ethicon, Somerville, NJ).
28. Isofl urane (Halocarbon Laboratories, River Edge, NJ).
29. Oxygen.
30. Sterile physiological saline.
31. 70% Ethanol.
32. Betadine.
33. Polysporin powder.
34. Antibiotics (e.g., Twin-Pen, Combi-Pen 48).

2.2. Motor Assessment via Grid Walk

1. Horizontal ladder elevated on a table (a set of 20 round, smooth, stainless steel bars, secured between two 2 in. × 4 in. pieces of wood in holes drilled such that the bars fit snugly. Each metal bar is 1.77 cm in diameter and the bars are spaced evenly every 3.43 cm on center).
2. 30.5 cm × 30.25 cm Black plastic box.
3. Stopwatch.

3. Methods

3.1. Avulsion Surgery

Two versions of the avulsion surgery are described, the first surgery is of shorter duration and used for the study of systemically administered pharmacological agents. The second surgery includes suturing the dura, reinstating an intact cerebrospinal fluid space. This second surgery was developed for intrathecal administration of pharmacological agents after the incised dura heals. In both cases, sham surgeries are carried out as described below with the exception that the roots are not avulsed. The first few steps are identical for both approaches and are noted below followed by the individual approaches. Following both surgeries, rats are transferred to their home cages fitted with cardboard boxes and foam padding to confine movement and protect it while recovering from anesthesia.

3.1.1. Common Surgical Steps for Both Models

1. Surgery is conducted under isoflurane anesthesia, 2.5% in oxygen. Isoflurane is chosen due to its minimal effects on immune cells compared to other anesthetics (13– 19).
2. The anesthetized rat is placed, dorsal side up, with the body lying parallel to the table's edge. The back is shaved, starting from mid rib cage and extending 7.5 cm caudally.
3. The exposed skin is cleaned with 70% ethanol.
4. The appropriate spinal level is identified using vertebral landmarks relative to T13, the rostral aspect of which is level with the 13th rib (the floating rib). A midline incision is made through the skin, extending 3.5 cm rostral and 3.5 cm caudal of this point. After opening the skin, the muscle is bilaterally separated as close to the vertebral dorsal processes as possible, by first slitting the fascia and underlying muscle down to the vertebral bone on each side of the dorsal processes and then scraping the dorsal processes and lamina with a #10 scalpel blade to clean remaining tissue from the bones. The midline tissue that “caps” the tops of the dorsal processes is removed, revealing the dorsal aspects of spinous processes of T11, T12, and T13.
5. The floating rib is used to identify the T13 dorsal process, and it is removed to improve access to the caudal aspect of T12 vertebral bone for laminectomy. Using toothed forceps to elevate and stabilize the vertebral column, rongeurs are used to remove the dorsal aspect of the T12 vertebra as well as the ipsilateral subarticular process of T13 and ipsilateral caudal end of T11, thereby exposing the dura-encased spinal cord. Care is taken to ensure that the laminectomy leaves no loose bone shards and no points of intact bone protruding toward the spinal cord as these could damage the spinal cord if swelling occurs in response to surgery. Dorsal roots T13 and L1 enter the spinal cord under the T12 vertebra.
6. Gelfoam sponge is teased apart into tiny pieces (~1.5 mm × 2 mm), and placed in a 500- μ L centrifuge tubes containing sterile physiological saline (see Note 1).

3.1.2. Avulsion Surgery Alone

1. The exposed dura is gently nicked using a 23-G $\frac{3}{4}$ in. hypodermic needle as a knife, by repeated gentle rostrocaudal strokes upon the dura parallel to midline of the spinal cord, but avoiding the dorsal midline blood vessel. The repeated exposure to the needle weakens the dura (see Note 2). When an opening appears, the hooked #5 forceps are used to very gently pick up the dura, and then open it using spring scissors such that an opening spans the opened vertebral space, and exposes T13 and L1 roots.
2. The dorsal roots are identified and individually isolated. L1 dorsal root enters the spinal cord at the caudal end of T12 vertebra, and T13 enters the spinal cord at the rostral end of T12 vertebra. These nerves are identified and traced to the dorsal root entry zone. The root, as it enters the spinal cord, is firmly grasped with fine-tipped curved forceps at the dorsal root entry zone and rapidly torn away from the dorsal horn. Successful avulsion disrupts the dorsal horn, and red dots of blood appear where the nerve previously had entered the spinal cord (Fig. 2).
3. After avulsion, saline-moistened gel foam is placed over the exposed spinal cord.
4. After draping the rat to avoid suture dragging through the rat's fur, the two sides of the muscle incision are gently pulled together and sutured using 3–0 silk suture, and the skin closed using 16-mm Michel clips.
5. Following surgery, rats are returned to their home cages to recover from anesthesia. The rat is placed in a foam-padded cardboard box, such as the box from a case of 1-cc syringes, cut down to allow the cage top to close. Foam padding is also placed in the cage, against the vertical walls for few hours to protect the rats from unintended spinal cord injury during the brief ataxic period associated with recovery from anesthesia (see Note 3).
6. Antibiotics (0.3 mL per rat) are administered at the time of surgery and then daily (0.2 mL per rat) for 4 days following surgery.
7. Avulsion surgery without dura suturing produces robust and reliable hindpaw mechanical allodynia compared to sham surgery 13–15 days postsurgery (see Notes 4, 6–9).

3.1.3. Avulsion Surgery with Dura Suturing

1. Upon completion of laminectomy, saline-moistened gelfoam is placed on the dura to keep it soft. The rat is then draped so to avoid suture from contacting rat fur. The drape extends beyond the rat's body so to provide a clean surface upon which the needed surgical tools are placed:
 - a. 6–0 silk, moistened by pulling it through a wet gauze pad to ensure that the suture is clean, flexible, and kink free. The tail end of the suture is grasped with a pair of hemostats, placed between the rat's body and the surgeon on the drape. This anchoring of the suture is to protect against

unintended tugs on the suture that can cause the surgeon to lose the suture placement in the dura.

- b.** Curved microforceps (#7 forceps), straight microforceps (#5 forceps) with a hooked tip, straight microforceps with no hooked tip, microscissors, 23-G Hypodermic needle.
- 2.** The exposure of dorsal roots is accomplished as two separate incisions: one caudally and the other one rostrally (see Note 2). This is necessary because the extreme challenge for dura suturing lies in the accessibility of the lateral dural edge for inserting the suture. As the incision gets longer, and as spinal cord swelling occurs (beginning as soon as the dura is first incised), access to the lateral dura edge rapidly degrades to impossible.
- 3.** After pushing the moistened gelfoam aside to expose the caudal dura, the 23-G hypodermic needle is used as a knife to gently cut a slit opening in the dura. Immediately grasp the needle of the 6-0 silk suture with the curved microforceps and, with the other hand, isolate and elevate the lateral cut edge of the dura with the hooked straight microforceps. Immediately insert the 6-0 silk through the lateral edge of the dura to gain a secure hold on the lateral dura. Note that it is extremely easy to tear the suture out of the lateral dura. All manipulation of the needle and suture must be with great gentleness. Hold a well-moistened (with either blood or saline) Q-tip gently atop the dura while pulling the suture through the dura to help prevent tearing. As blood makes it stick to the microforceps (used to pull the suture through), be very aware of this so that the stickiness does not result in unintentional pulling on the dura. Pull the suture through until restrained by the far end of the suture being held by the hemostats. Remove the moistened Q-tip and lay the needle end of the suture next to the hemostat, thus moving the suture out of the surgical field during the avulsion.
- 4.** The dorsal roots are identified and individually isolated, as described above (Subheading 3.1.2, step 2). Using #5 forceps, the nerves are trace to the dorsal root entry zone, grasped tightly with the forceps, and rapidly torn away from the spinal cord.
- 5.** Upon completion of the avulsion, the medial edge of the dura is elevated with the hooked straight microforceps, the needle of the 6-0 silk inserted though, a moistened Q-tip used to help prevent tearing of the dura by the suture as it is pulled through. Unclasp the tail end of the suture from the hemostat and complete the pulling of the suture so that a short tail of suture remains beyond the lateral dura. Gently tie the suture using fl at (not hooked) microforceps, and trim with microscissors. Clean the 6-0 suture with a saline-moistened gauze pad to remove the sticky blood and insure there is not tissue stuck to the suture that would tear the dura when again used to suture dura.
- 6.** Gently move the rostral gelfoam caudally over the just-sutured caudal site, thereby exposing the rostral section of dura-encased spinal cord. Repeat steps 3–5.

7. Following dura suturing, moistened gel foam is gently placed to cover the entire exposed spinal cord, and the muscle wall and skin are sutured in layers as described above (Subheading 3.1.2, step 4).
8. Rats are returned to their home cage with foam padding, as described (Subheading 3.1.2, step 5).
9. Antibiotics (0.5 mL per rat) are administered at the time of surgery then daily (0.4 mL per rat) for 4 days following surgery.
10. Avulsion surgery with dura suturing produces robust and reliable hindpaw mechanical allodynia compared to sham surgery 15–20 days postsurgery (see Notes 4, 5, 7–9).

3.2. Motor Assessment via Grid Walk

1. The ladder is placed on a well-lit, stable table and a black plastic box were placed at one end to encourage animals to travel in one direction across the ladder (towards the box, Fig. 3).
2. Rats undergo three habituation periods during which they are trained to walk across a horizontal ladder, to the black box.
3. For all baseline and postoperative testing, animals are required to walk across the ladder three consecutive times without stopping. If they stop on the ladder, they are gently prodded to continue.
4. Baseline measures are collected the day before surgery. Following surgery, rats are tested weekly.
5. Dependent measures used for analysis are the time needed to cross the ladder and the total number of hind-limb footfalls (missteps) for each trial. The time or number of missteps is averaged and used for analysis. Ipsilateral and contralateral hindpaw data are analyzed separately.

4. Notes

1. These surgeries can be initially time intensive, and resulting animal behavior is extremely sensitive to the amount of time the spinal cord is exposed. If there are environmental distractions that need to be dealt with, moistened gel foam can be placed on exposed cord with dura intact for a short amount of time, and the surgery continued with the expected results. If the dura has been cut, for a successful surgery, the remaining steps should be completed as seamlessly as possible. Once the dura has been cut, the spinal cord begins to swell, and continues to change for the duration of exposure.
2. A significant challenge with this surgery is completing it without otherwise damaging the spinal cord or local blood vessels. The key to successfully avulsing the dorsal roots and not nicking a blood vessel or otherwise injuring the spinal cord is that many of surgical actions are carried out in synchrony with the animal's breathing. Laying on a hard surface, the spinal cord moves up vertically

as the animal breathes, and thus facilitates forcep tips or needles unintentionally penetrating the parenchyma. When slitting the dura, it is optimal to make each cutting motion on the rat's exhale as at this point there is a pause in the movement of the spinal cord with respiration.

3. Following surgery, the rats are extremely vulnerable to further injury to the spinal cord, and as such care is taken when handling them and in housing them following surgery. Immediately following surgery, the rat is returned to its home cage containing a box with foam padding along the vertical walls and floor to confine movement during anesthetic recovery. The padding is removed once the animal completely recovers. Food is placed directly in the cage and the water bottle has an extended spout, all done to prevent the rat from further injury to the spinal cord. Additionally, the rats are handled with extreme care, always using two hands to fully support the rat and minimize struggling.
4. Following a successful avulsion surgery, sham- and avulsion-operated rats show hindpaw allodynia over the first 2 weeks, at which point the behavior of the sham operated animal approaches baseline levels, with sham-operated rats completely returned to baseline by 3 weeks. During the first 2 weeks, avulsion-operated rats, with or without dura suturing, show some paresis in the left hindpaw. Recovery from surgery is marked by loss of paresis and when there is no difference between naïve, sham-operated and avulsion-operated rats with respect to movement across the horizontal ladder. Hindpaw mechanical allodynia is induced 100% of the time with successful avulsion of both T13 and L1 dorsal roots. Approximately 4% of the rats show autotomy (evidenced by the development of sore on or near the ipsilateral knee) during the first 3–4 weeks, and resolves in about a week. We have treated the autotomy with antibiotics and have not found that it interferes with hindpaw allodynia. When the spinal cord and/or surrounding blood vessels are injured or if the forceps slip into the cord during surgery, usually one or both hindpaws are paralyzed, and the animal does not completely recover from surgery.
5. Successful dura suturing can be tested after the animal has recovered from the surgery by co-administering Evan's blue and lidocaine intrathecally to the injury site. Lidocaine paralyzes the animal at the dermatomal level it is administered, and as such serves as a functional measure to the viability of an intrathecal injection. The paralysis is evident as soon as the animals recover from anesthesia. Thirty minutes after the intrathecal injection and behavioral verification, the animal is deeply anesthetized and transcardially perfused with saline to facilitate visualization of the dye. If the surgery with dura suturing is successful, the Evan's blue dye will remain within the cerebro-spinal fluid space around spinal cord, will not be found outside of the dura indicative of a leak. In our experience, intrathecal injections were successful 5 days postsurgery for avulsion with dura suturing.
6. Histological confirmation of successful dorsal root avulsion can be obtained by cresyl violet staining, and by taking advantage of the interaction between 3,3-

diaminobenzidine (DAB; Sigma-Aldrich) and endogenous peroxidases expressed by red blood cells. Spinal cord slices are reacted using DAB for 15 min, and glucose oxidase (Sigma-Aldrich; type V-s; 0.02%) and β -D-glucose (0.1%) are used to generate hydrogen peroxide. Nickelous ammonium sulfate is added to the DAB solution (0.025%, w/v) to intensify the reaction product. Slides are then dried overnight, cleared, and coverslipped with Permount. Successful avulsion readily shows influx of red blood cells 24 h after surgery. With cresyl violet staining, one can visualize the tissue disruption caused by the avulsion.

7. When first learning this surgery, a surgeon can expect to complete the avulsion alone surgery within 75 min. Once one becomes expert at the avulsion alone surgery (which now takes about 20–25 min), first learning the avulsion with dura suture surgery extends the surgical time to about 60 min. This decreases to about 40–45 min with expertise. As already discussed, the challenge is not injuring the spinal cord other than that caused by avulsing the dorsal roots. A high-percentage loss when learning the dura suturing surgery stems from a combination of factors including inability to secure the suture through the lateral dural edge, tearing of the dura while avulsing the roots, etc. With practice, the surgery can be done in about 40 min with rare loss of rats. There will, however, be rats whose blood vessel patterns make it impossible to do an avulsion without having a blood-obscured surgical field. These rats can be assigned as sham-operated controls or discarded, whichever is preferred.
8. Hindpaw mechanical allodynia is induced 100% of the time with successful avulsion of both T13 and L1 dorsal roots. The time a novice surgeon requires to complete the surgery potentiates the pain, which is observed in sham-operated rats such that the sham rats are indiscernible from avulsion rats for at least 2–4 weeks. The time to complete surgery is significantly shortened with experience, and the behavioral profile of sham- and avulsion-operated rats differs significantly within the first 2 weeks following surgery.
9. As this chapter is being written, we are optimizing methods for the measurement of at-level pain. We have been successful at detecting trunk allodynia in 1 and 2 ipsilateral dermatome levels above and below T13 and L1, and these same dermatomes on the contralateral side, as well as at T13 and L1. The animal is transferred in its home cage to a dimly lit, quiet behavioral testing room. All testing takes place in the animal's home cage, and is measured by the number of times out of 10 that the animal responds to the 4.56 (3.363 g) von Frey filament per side, each stimulation lasting up to 3 s. Baseline measures are collected the day before surgery, and weekly testing begins 2 weeks after surgery. A behavior is determined to be avoidant if the animal either bit at the filament, jumped away, escaped to another area of the cage, or vocalized.

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Fig. 1. Photograph generally illustrating the location of injury relative to the level the pain is experienced. Avulsion of both thoracic (T)13 and lumbar (L)1 dorsal roots induces mechanical allodynia four dermatomal levels (L5/L6) below the site of injury, measured in the hindpaw.

Fig. 2.

Photograph of spinal cord immediately following dorsal root avulsion. Rats undergo laminectomy and retraction of the dura, exposing the dorsal roots entering the spinal cord. The dorsal roots are individually identified, isolated, and rapidly avulsed from the spinal cord. This avulsion disrupts the dorsal horn, leaving holes in the spinal cord at the point the roots entered the spinal cord. Modified from Wieseler, et al. (20) with permission from Mary Ann Liebert, Inc.

Fig. 3.

Photograph of horizontal ladder used to assess motor ability in the grid walk. **(a)** The horizontal ladder has metal rungs spaced 2.54 cm apart. The ladder consists of 20 rungs, with only the middle 10 being used to test behavior, that way the behavioral measurement is not affected by the first uncertain steps, or the last rushed steps. **(b)** With habituation, a naïve rat rapidly and effortlessly moves across the horizontal ladder.