In Vivo Electrophysiological Recording Techniques for the Study of Neuropathic Pain in Rodent Models

Fei-Yue Zhao,[1] Ross Jeggo,[1] Haifeng Wei,[1] Andrew Whyment,[1] Xin Fang,[1] and David Spanswick[1,2,3]

[1]NeuroSolutions Limited, Coventry, United Kingdom
[2]Department of Physiology, Monash University, Clayton, Victoria, Australia
[3]Warwick Medical School, University of Warwick, Coventry, United Kingdom

ABSTRACT

Neuropathic pain develops following nerve injury, and is a chronic pain syndrome that can persist long after repair of a wound or removal of the neurological insult. This condition remains poorly treated, not least because of a lack of mechanism-based therapeutics. Clinically, neuropathic pain is characterized by three major symptoms: thermal or mechanical allodynia (pain sensation in response to previously non-noxious stimuli); hyperalgesia (enhanced pain sensation to noxious stimulation); and spontaneous, ongoing pain. These clinical symptoms can be modeled in rodent neuropathic pain models using behavioral and electrophysiological readouts. This unit describes techniques designed to record pathophysiological electrical activity associated with neuropathic pain at the level of the periphery, in single fibers of primary sensory neurons, and from wide dynamic range (WDR) neurons of the dorsal horn of the spinal cord. These techniques can be employed in both naïve animals and in animal models of neuropathy to investigate fundamental mechanisms contributing to the neuropathic pain state and the site, mode, and mechanism of action of putative analgesics. *Curr. Protoc. Pharmacol.* 66:11.15.1-11.15.26. © 2014 by John Wiley & Sons, Inc.

Keywords: electrophysiology • peripheral nerve • ectopic discharge • spinal cord • dorsal horn neurons • neuropathic pain

INTRODUCTION

Chronic pain, associated with abnormal levels of electrical excitability of the somatosensory nervous system, represents a significant medical challenge and an unmet health and socioeconomic burden. Estimates suggest that ~33% of Americans and 25% to 30% of Europeans experience chronic pain, and of these cases, 15% to 25% reflect pain of neuropathic origin (Leadley et al., 2012). Primary lesions and dysfunction of the nervous system, including diabetic and cancer chemotherapeutic–induced neuropathy, post-herpetic neuralgia, certain types of neurological degenerative disorders, and stroke can all initiate neuropathic pain (see Cohen and Mao, 2014). Nerve injury accompanied by exaggerated responses to painful stimuli (hyperalgesia), painful responses to previously innocuous stimuli (allodynia), abnormal spontaneous sensations (dysesthesia), and a feeling of "burning" pain in the absence of stimuli characterize the neuropathic pain state. Generally, neuropathic pain remains poorly treated due to a lack of mechanism-based therapeutics, reflecting a lack of understanding of the underlying mechanisms, coupled with the fact that these mechanisms may differ depending on the initiating insult, its duration, and time post insult. As with all chronic pain states, neuropathic pain is associated with a remarkable and complex plasticity within the nervous system—nerve injury leading to changes in gene expression as well as ion channel and receptor function in neurons, glia, and microglia, along with functional rewiring, sensitization of the neural pathways, and changes in neuronal excitability (Basbaum et al., 2009;

Published online September 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471141755.ph1115s66

Latremoliere and Woolf, 2009). Peripheral neuropathic pain is characterized by spontaneous action-potential firing in primary sensory afferents. This ectopic discharge or firing in normally quiescent primary sensory neurons is considered the principal drive for pain in neuropathy, functioning as a "pain" signal and driving central sensitization. Consequently, recognizing the mechanisms underlying ectopic firing and central sensitization are key to designing and validating future therapeutic approaches to target this debilitating condition. This unit describes, in detail, electrophysiological methods for recording spontaneous ectopic discharge from peripheral nerves in rodent models of neuropathic pain. We also describe the methods employed to record activity in wide dynamic range (WDR) neurons in the spinal cord. These second-order neurons process both nociceptive and non-nociceptive information from the periphery, and are subject to sensitization in neuropathic pain states.

Neuropathic Pain

Neuropathic pain is modeled in rodents in several different ways, including injury to primary sensory nerve axons, such as the sciatic nerve ligation or chronic constriction injury model (CCI model; Bennett and Xie, 1988); spinal nerve ligation (SNL or Chung model; Kim and Chung, 1992); partial sciatic nerve ligation (PSL or Seltzer model; Seltzer et al., 1990); axotomy (Wall and Gutnick, 1974); and dorsal root ligation or section. Other models in which neuropathic pain is a co-morbidity and of clinical relevance include models of diabetes (spontaneous diabetes model, Burchiel et al., 1985; streptozocin (STZ)-induced diabetes model, Khan et al., 2002; *UNIT 5.47*) and neuropathy induced by chemotherapeutics like cisplatin, the taxanes, and thalidomide (Xiao et al., 2012). All of these models are characterized by ectopia (spontaneous activity; see Background Information), although whether the mechanisms underlying the generation of this spontaneous activity are the same across all models is unclear and mechanistically highly unlikely. What is clear is that ectopic firing in neuropathic pain can be generated in both damaged and neighboring intact, nociceptive and non-nociceptive, C, Aδ, and Aβ primary afferent neurons (Devor, 2009; Djouhri et al., 2012; Zhu et al., 2012), and can be generated at the level of the soma in the dorsal root ganglion, the neuroma formed at the site of injury, the axon, or the peripheral nerve terminals themselves (Campbell and Meyer, 2006; Schaible, 2007). Electrophysiological recording of ectopic discharge in vivo permits characterization and classification of the ectopic discharge itself, the sensory afferent generating activity (Aβ, Aδ, C-fibers; based upon conduction velocity), and, through stimulation of the receptive field, the sensory modality (Han et al., 2000; Zhao et al., 2007; Hopkins et al., 2013). The response of fibers to mechanical, thermal, chemical, and electrical stimulation can be compared between disease-model and naïve animals. Furthermore, the power of this technology is most clearly illustrated when it is used to identify the site of origin of ectopic firing through recording activity in surgically isolated sections of the pathway, and, in a similar manner, the site of action of existing or novel therapeutics. Such information is not only valuable for understanding the site, mode, and mechanism of action of a therapeutic, but also for informing chemists designing these agents.

WDR neurons, also termed convergent or multi-receptive neurons (Mendell, 1966), are located in the dorsal horn of the spinal cord, the thalamus, and the cortex. They receive nociceptive synaptic input from slowly transmitting Aδ and C fiber primary afferents as well as non-noxious somatosensory input from faster-conducting Aβ fibers. They respond relatively linearly, with small responses to innocuous stimuli—e.g., light touch—rising to increased responses as the stimulus intensity of their peripheral receptive field reaches into the noxious range, e.g., sharp pinch. WDR neurons also display a phenomenon termed "wind-up" (Herrero et al., 2000), in which they demonstrate a frequency-dependent increase in excitability, induced by repeated electrical stimulation of peripheral afferents.

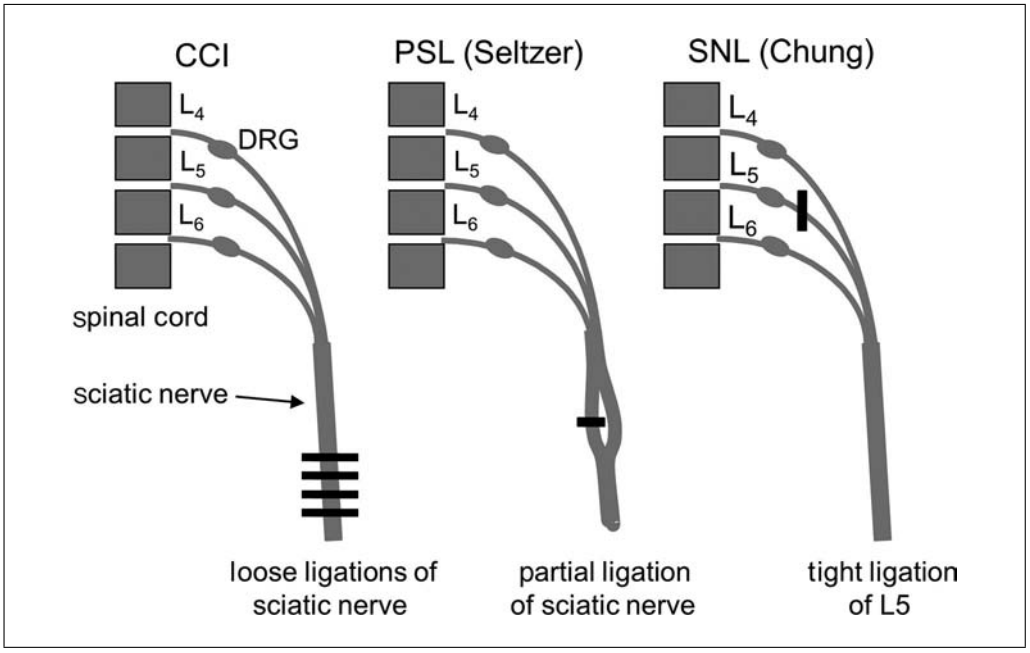

Figure 11.15.1 Schematic diagram illustrating the site of nerve ligation for three commonly used surgically-induced neuropathic pain models: CCI, PSL, and SNL. L4-L6 refer to L4 to L6 spinal nerves. Black blocks represent site of nerve ligation.

Wind-up is an experimental manipulation that induces a central "sensitized" state, and can be achieved in both animal models and human volunteers (Woolf, 2011). Similar to peripheral single nerve fibers, under normal resting conditions WDR neurons are relatively quiescent, with few spontaneous action potentials. However, after repeated electrical stimulation (e.g., wind-up) or following nerve injury induced in rodent models of neuropathic pain, WDR neurons usually show increased spontaneous firing, a sensitized response to low-intensity (normally innocuous) stimulation (allodynia), and an enhanced response to high-intensity stimulation (hyperalgesia). Consequently, they are among the most widely studied neurons for examining the mechanisms that underlie the development of chronic pain states, and are therefore also highly suitable for testing novel peripherally or centrally acting pain therapeutics.

Neuropathic pain models

Listed below are descriptions of the intervention required to induce a number of different neuropathic pain models to help the reader have some understanding of their background, how they are induced, and their application in either mechanistic investigations or compound testing. For further detail on the method of model induction and associated validation, refer to *UNIT 5.32* (CCI, PSL, SNL models), *UNIT 5.47* (STZ-induced diabetic neuropathy), or the original publications, where listed. Figure 11.15.1 shows a schematic diagram of the surgical manipulation performed in three commonly used neuropathic pain models.

1. The chronic constriction injury model (CCI, Bennett and Xie, 1988, *UNIT 5.32*) is a model of mononeuropathy induced by placing three to four loose ligations on either the left or right sciatic nerve. The damage to the peripheral nerve involves infiltration by inflammatory cells (e.g., mast cells, macrophages and lymphocytes) that secrete cytokines to stimulate an initial predominantly inflammatory phase of injury (<2 weeks), followed by a subsequent predominantly neuropathic phase of injury 2 or more weeks after surgery. Successfully modeled animals develop mechanical allodynia (PWT $\leq$ 4.0 g), assessed by their sensitivity to a series of graduated von Frey hairs, and thermal hyperalgesia, assessed using the plantar test (Hargreaves'

method), and can be used for electrophysiological recording experiments investigating sensitized pain responses and ectopic discharge.

2. The partial sciatic nerve ligation model (PSL/PNL, Seltzer et al., 1990; *UNIT 5.32*) is a similar model of mononeuropathy to the CCI model, with surgery taking place at the same site. However, in this case 33% to 50% of the dorsal aspect of the sciatic nerve is isolated before tight ligation, leaving the remaining nerve uninjured. The PSL model is regarded as having a reduced inflammatory component compared to the CCI model, although the time taken for neuropathy to develop is the same, at 2 weeks, and the pain behavior, validation method, and experimental application are consistent between models.
3. The spinal nerve ligation model (SNL, Kim and Chung, 1992; *UNIT 5.32*) is a robust and highly reproducible model of neuropathy that simulates "causalgia," or complex regional pain syndrome. It is surgically induced by tightly ligating spinal nerves L5 and L6, or L5 alone, distal to the dorsal root ganglia. The SNL model mimics both acute and chronic neuropathic states found in man, with animals developing mechanical and cold allodynia that persists for up to 7 weeks or longer and that can be used for electrophysiological investigation of sensitized pain responses and ectopic firing.
4. A diabetic neuropathy model can be induced chemically in rodents by the intraperitoneal injection of streptozotocin, which selectively destroys pancreatic β-cells (*UNIT 5.47*). This model of polyneuropathy induces diabetic symptoms, e.g., polydipsia, polyphagia, and polyuria, that become significant after 3 to 4 days. Within this time period, the animals also become hyperglycemic, with blood glucose levels as high as 20 to 30 mM or more. The model is characterized by a reduction in nerve conduction velocity and altered sensory sensitivity, and mechanical allodynia can be observed 2 to 4 weeks after induction—the optimum time for electrophysiological investigation of pathological activity.
5. A chemotherapy-induced neuropathy model (Xiao et al., 2012) can be induced by the administration of commonly used anti-cancer agents, such as paclitaxel (4 to 5 mg/kg, i.v./i.p. twice a week for up to 5 weeks), oxaliplatin (3 mg/kg, i.v./i.p., twice a week for up to 4 weeks), and vincristine (0.1 mg/kg, i.v./i.p. for 5 days a week for 2 weeks). Similar to diabetic-induced neuropathy, this model of polyneuropathy is characterized by damage to the nerve fibers, and can be validated by assessment of mechanical allodynia using von Frey hairs. After the development of neuropathy, treatment with the chemotherapy agent can be halted and the resultant pain behavior and neural sensitization will persist for 2 weeks or more, allowing electrophysiological investigation into this modeled condition.

Technological advancement

Although the fundamentals of the electrophysiological approach outlined in this protocol have remained relatively consistent for decades, technological advances in electronics and equipment used for such studies are in a constant state of evolution. Incorporating the latest hardware and software is an important component to ensure maximum quality and quantity of data acquisition at every stage. Thus, while the protocols described here serve as a template for carrying out electrophysiological investigation into peripheral nerves and spinal cord neurons in intact animals, adaptability and flexibility are critical for the design and implementation of appropriate and relevant electrophysiological investigations.

Pharmacokinetic considerations

When compounds are being evaluated in vivo, it is imperative that some preliminary pharmacokinetic data be generated to ensure that the compound is known to be present at the time of testing and in an adequate amount. Increasingly, in vivo studies rely more on the measured plasma level of a compound, rather than the dose administered,

to construct accurate dose-response curves. For peptides, a half-life of less than 1 min can be incompatible for a test procedure where animals are measured for behavior or a performance phenotype 30 or 60 min after compound administration. However, many compounds can produce their effects via alterations in gene expression, which can be long lasting, such that their biological half-life is many times longer than the actual presence of the compound in the plasma. If a short-acting compound produces an effect beyond its plasma half-life, this can provide valuable information on its potential mechanism of action. Conversely, a behavioral effect of a compound that parallels its plasma half-life implies a direct cause-and-effect relationship that is proportional to the plasma concentration. If there is no pharmacokinetic information on a compound, this can seriously compromise the intent and outcome of the experiment.

Protocols in this Unit

Basic Protocol 1 describes techniques to record normal, mechanically evoked, and sensitized activity from fibers of the sciatic nerve that have been classified by their conduction velocity in CCI model rats, while Alternate Protocol 1 provides variations on these techniques that enable investigation into other components of the sensory pathway within the peripheral nervous system. Basic Protocol 2 describes techniques to monitor the extracellular activity and properties of WDR neurons located within the dorsal horn of the spinal cord, in naïve and neuropathic pain–model rats, while Alternate Protocol 2 describes how it is possible, with surgical intervention, to remove the impact of higher centers and the peripheral nervous system from this investigation.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

MEASUREMENT AND CHARACTERIZATION OF SPONTANEOUS ECTOPIC DISCHARGE RECORDED FROM PERIPHERAL NERVE BUNDLES OF THE SCIATIC NERVE IN NEUROPATHIC MODEL RATS

BASIC PROTOCOL 1

Described in this protocol are basic procedures for monitoring the frequency and firing patterns of spontaneous ectopic discharge recorded from nerve filaments of neuropathic pain–model anesthetized rats, as well as measurement of their conduction velocity using a routine stimulation-response protocol (Fig. 11.15.2). These procedures are applicable in many rodent models of neuropathic pain after behavioral validation of the pain state (see *UNIT 5.32* and the Commentary in this unit). Specifically, this protocol describes procedures for fiber recordings from the sciatic nerve of CCI model rats and alternate steps that describe modifications of these procedures to permit monitoring ectopic discharge from a number of other sites within the neuraxis (see Fig. 11.15.2), which may be employed for a variety of related purposes.

Materials

Sprague-Dawley rat (male, 300 to 400 g; neuropathic pain state behaviorally validated, see Commentary; also see *UNIT 5.32*)
12% urethane (see recipe)
Normal saline (NS, see recipe)
Heparinized normal saline (see recipe)
Superglue
Mineral oil
Test substances, e.g., lidocaine, mexiletine, or gabapentin (see recipes)
Pentobarbital sodium (see recipe)

Syringe pump (Harvard Apparatus; optional)

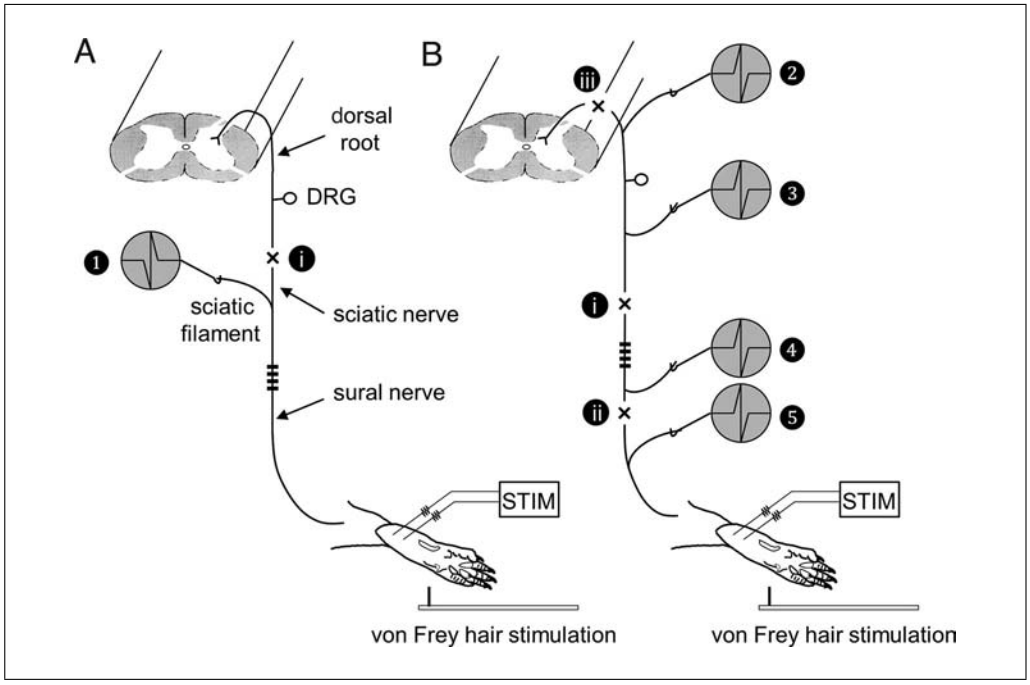

Figure 11.15.2 Schematic diagrams showing the setup for single-fiber recording from a CCI model rat. (**A**) Recordings can be made from (1), sciatic nerve fiber proximal to the ligation area, with section at (i), to record the activity generated from the neuroma and/or from more peripheral axons or the receptive field. (**B**) Recordings can be made from site (2), dorsal root filaments proximal to the dorsal root ganglion (DRG), with sections at (i) and (iii), to record activity generated from the DRG. Site (3), sciatic nerve fibers distal to the dorsal root ganglia (DRG) for the recording of the activity generated from the DRG. Site (4), sural nerve fibers distal to the ligation area with section at (i) and (ii) to record neural activity of neuroma origin. Site (5), sural nerve fibers connected with the peripheral receptive field and sectioned proximally at (ii), to record spontaneous and evoked activity from axons close to the receptive field on the hind paw. The four black bars indicate the four loose ligations placed on the sciatic nerve to generate the CCI model.

Animal clippers
Homeothermic blanket system and rectal probe (Harvard Apparatus)
Polyethylene tubing (external/internal diameter 0.96/0.58 mm) for cannulation of jugular vein/carotid artery
26-G needles
Three-way tap
Recommended surgical instruments:
- Scalpel no. 3 with blade no. 10 or no. 11
- Hemostatic clamp, curved
- 15-cm large operating scissors
- 8-cm medium dissection scissors
- Large blunt-head dissection forceps, curved
- Small blunt dissection forceps, curved
- Fine forceps (No. 5 or 55; two are required)
- Small arterial clip (micro clamp)
- Iris scissors, 5.7 cm, curved
- Suture needles (size 0 and 1)
- Needle holder
- Silk suture (4/0 or 3/0)
- Bone rongeur with extra-fine tips (Friedman-Pearson via FST)

Hemostatic gelatin sponge
Absorbent cotton wool balls
Pressure amplifier and transducer (Digitimer, cat. nos. NL108 and NL108T2)

Analog/digital converter (Micro1401, Cambridge Electronic Design)
Computer running Spike2 software (Cambridge Electronic Design)
NeuroLog modular acquisition hardware (NeuroLog, Digitimer, cat. no. NL905)
A.C. preamplifier and filter modules (Digitimer, cat. nos. NL104 and NL125)
Metal O-ring frame (6 cm diameter)
Stereomicroscope
Fiber-optic light source
Opaque plastic platform (~5 mm × 10 mm)
Temper-annealed silver wire (Ø, 0.37 mm, Advent Research Materials Ltd., cat. no. AG549711)
Headstage (Digitimer, cat. no. NL100AK)
von Frey hairs (1 g, 4 g, and 15 g; Stoelting, cat. no. 58011)
Isolated constant-voltage stimulator (DS2A, Digitimer)
25-G syringe needles (Becton Dickinson, cat. no. 300600) connected via insulated copper wires to 4 mm plugs

Additional reagents and equipment for injection of rats (Donovan and Brown, 2006)

Prepare for surgery

1. Anesthetize a rat with 12% urethane at a dose of 1.2 g/kg i.p. (e.g., for a 300 g animal, use 3 ml). Administer 0.2 g/kg supplementary anesthetic either i.p. (pre-cannulation) or i.v. (post-cannulation) as necessary to ensure adequate anesthesia. Assess the level of anesthesia by monitoring the response to a paw-pinch (withdrawal reflex) and the stability of cardiovascular variables (post-cannulation).

   Frequent anesthetic assessment is imperative for animal welfare and stability of the preparation.

   Animal injection techniques are described in Donovan and Brown (2006).

   Alternative anesthetics can be used, such as sodium pentobarbitone (Sigma; 60 mg/kg, i.p.), followed by 6 to 20 mg/kg/hr, i.v. via a syringe pump (Harvard Apparatus), or thiobutabarbital sodium (Inactin), 120 mg/kg, i.p., followed by 20 to 30 mg/kg/hr, i.v.

2. Shave the ventral aspect of the neck from the clavicle to the jaw, and the lateral aspect of the left hindlimb from the ankle to the pelvis.

3. Maintain animal core body temperature at 37°C by placing the anesthetized subject on a heating blanket system connected to a rectal probe.

4. Cannulate the right jugular vein and carotid artery for drug administration and arterial blood pressure recording with 0.96-mm external diameter tubing prefilled with normal saline (NS) and heparinized NS, respectively, and fitted on the free end with a similarly prefilled 26-G needle and three-way tap.

Cardiovascular variable monitoring and animal positioning

5. Connect the carotid artery cannula to the pressure transducer prefilled with heparinized NS using the three-way tap and begin monitoring arterial blood pressure via the pressure amplifier, Micro1401 data acquisition system, and computer running Spike2 software.

   To optimize the arterial blood pressure signal, it is essential that no tight bends be present in the cannula tubing and no air bubbles be present in the needle, tubing, three-way tap, or pressure transducer.

6. Fix the left hind paw to a metal platform with superglue, with the dorsal surface facing downward, in a natural functional position leaving the paw plantar surface exposed for receptive field exploration and stimulation.

Fixing the paw to a platform restricts the movement of the ankle joint to avoid movement-induced activity (from proprioceptive cells) when examining the receptive field of selected fibers with either a light brush or von Frey hairs (e.g., 6 g). Also, this step helps to ensure that the recorded neural activity is from axons of the sural nerve, which is a 'pure' sensory nerve with most receptive fields located on the plantar surface.

Perform nerve dissection and fiber-teasing

7. Make an incision, parallel to the long axis, on the lateral aspect of the hindlimb, and separate the skin from the sub-dermal tissues.

8. Stitch the skin flaps onto a metal O-ring to form a pool.

9. Crush the surface muscle with a pair of hemostatic forceps to avoid hemorrhage, and remove carefully to expose the sciatic nerve.

10. Cover the nerve with warm (~37°C) mineral oil, to prevent the tissue from drying out.

11. Using a stereomicroscope and a fiber-optic light source, separate the sciatic nerve from the surrounding connective tissue (see Fig. 11.15.2A) about 1.5 to 2 cm proximal (i.e., closer to the spinal cord/central nervous system) to the nerve ligation injury area (in the CCI model animal, approximately mid-thigh level), being careful not to damage the nerve.

12. Section the sciatic nerve as proximally as possible, usually around the greater trochanter, to facilitate fiber teasing and fiber-type categorization (determination of conduction velocity). Place the distal-originating sectioned end (i.e., closer to the peripheral end of this neural pathway; the receptive field) onto an opaque plastic platform.

    The longer the distance between stimulation and recording sites, the better, as this aids in the identification of faster conducting fiber types, e.g., Aβ fibers, by giving a longer latency of response, separating it from any stimulus artifact that may be present.

    The opaque plastic platform (~5 mm × 10 mm) is useful to allow clear visualization of the nerve and dissected bundles during fiber teasing when viewing under the light microscope, providing greater contrast.

Fiber recording

13. Carefully remove approximately 0.5 cm of the nerve sheath (epineurium) from the cut end using iris scissors and fine forceps.

    Removal of the epineurium (de-sheathing) is a crucial step for the subsequent fiber teasing and recording, as incomplete or poor de-sheathing will reduce the number of live fibers and affect the recording quality.

14. Using fine forceps, repeatedly tease and split apart the nerve into fine bundles, then loop one bundle at a time onto the unipolar silver wire recording electrode to allow assessment of spontaneous single fiber activity, e.g., as shown in Figure 11.15.3.

    So-called 'single fiber recording' in reality means 'small bundle recording': recording from a fine 'bundle' containing several live fibers, among which are only one or two fibers with spontaneously occurring action potentials. In this protocol, all 'single-fiber' and 'filament' references equate to 'fine bundles'.

15. Prepare for recording unitary activity by connecting the recording electrode to the 'A-In' socket of a recording headstage, and the 'B-In' and 'GND' sockets of the headstage to the metal O-ring (see step 8) via an insulated copper wire to form a reference electrode. Connect the headstage to the amplifier with the gain set to 5 K and the parallel filter low/high frequency pass set to 0.5/5 kHz, respectively (see Fig. 11.15.4).

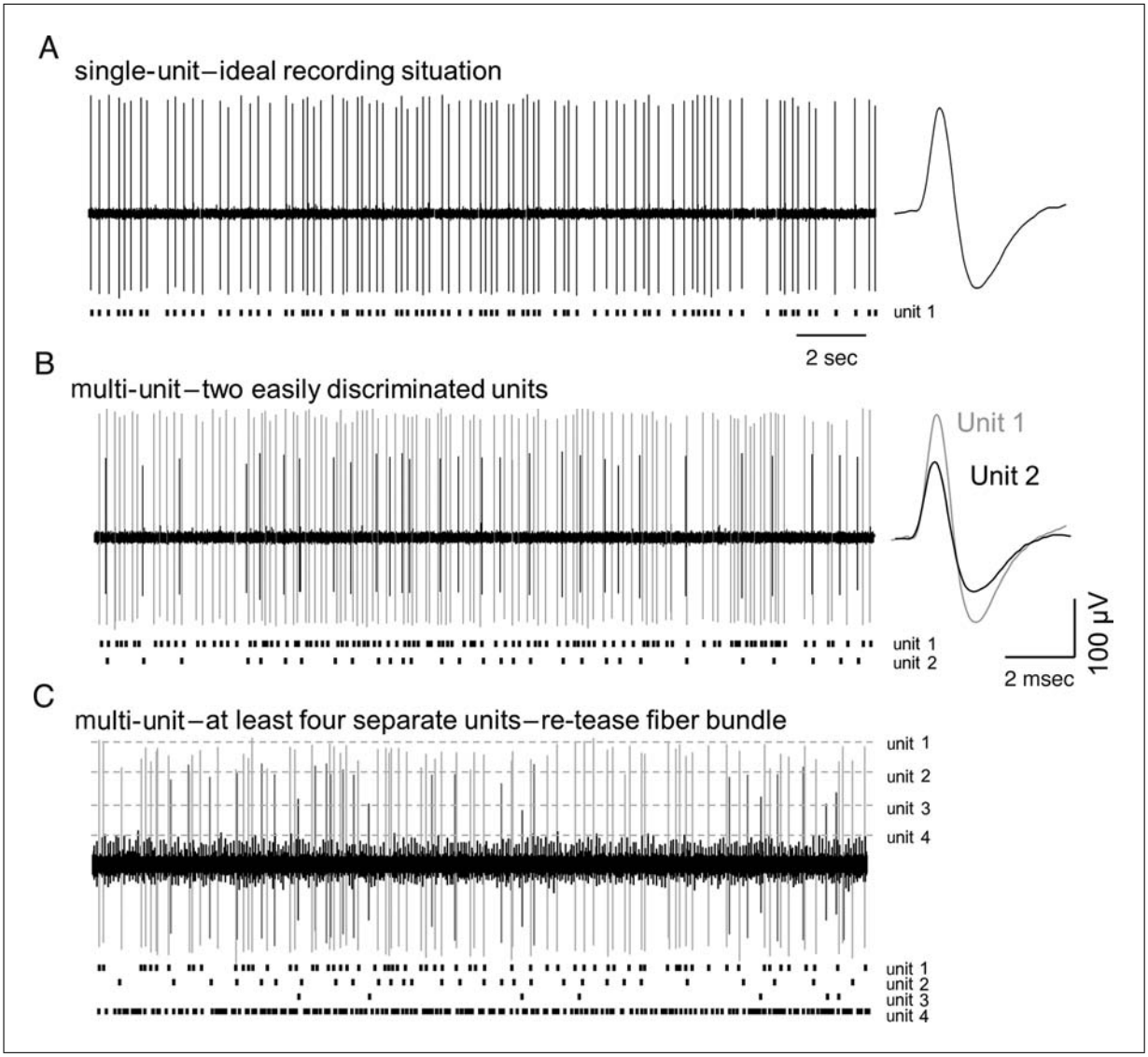

Figure 11.15.3 Example recordings of spontaneous ectopic discharge from a neuropathic pain model rats illustrating sciatic nerve bundles with either a single unit (**A**), two clearly definable units (**B**), or multiple units (**C**). The trace shown in (A) is the ideal recording situation containing a single unit, as seen expanded on the right-hand side. Trace (B) shows a recording with two separate units that can be easily discerned and discriminated using the recording software; again, each unit can be seen on an expanded scale on the right. Trace (C) shows a recording containing multiple units (at least four) of similar amplitude, which would be difficult to discriminate manually or using the recording software. In this situation, further teasing of the fiber bundle is recommended to reduce the number of individual units.

To maintain the highest possible signal-to-noise ratio for recorded responses, connect any items that are likely to produce electrical noise (e.g., the fiber optic light source) to a central earthing point, along with any large metallic items (e.g., the vibration isolation table). A floor- or table-mounted Faraday cage will also help minimize electrical noise.

16. Record the spontaneous activity from the selected fine bundle using Spike2 software, and either proceed with the recording if the quality is suitable, replace the bundle with another, or remove this bundle and tease apart further to improve the quality (see Fig. 11.15.3).

    It is possible to proceed with recordings with two active fibers if they can be easily discerned based on their amplitudes and their activity is stable.

17. Once a fiber with stable spontaneous activity has been identified, locate the receptive field for this fiber on the hindlimb. This is achieved using a brush, a von Frey hair,

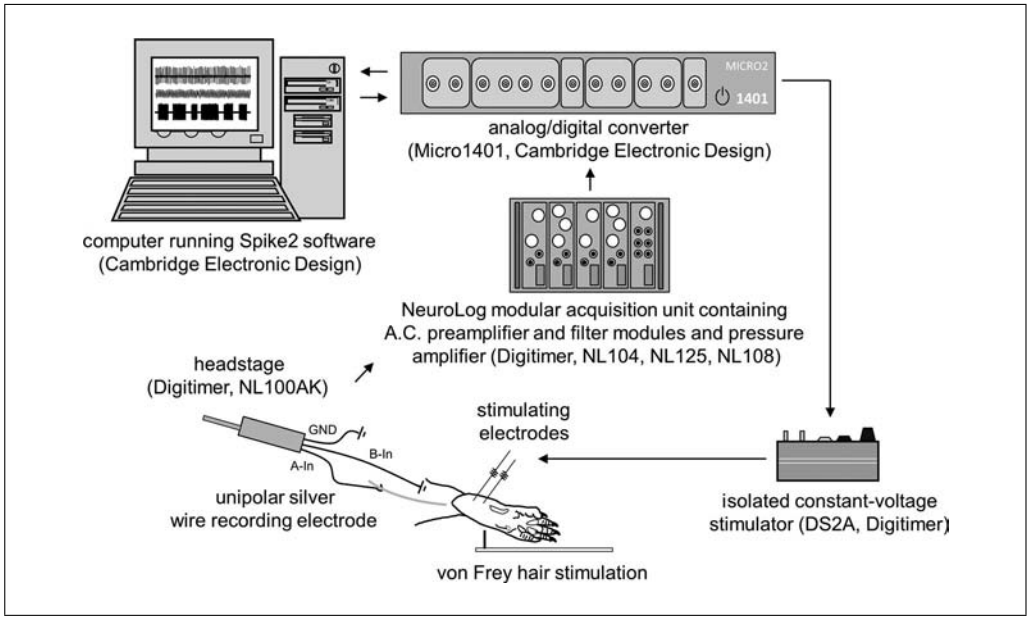

Figure 11.15.4 A schematic diagram that shows the connections between the various elements required to perform a single-fiber recording experiment. This is similar to the setup required for recording spinal cord dorsal horn neurons, with the exception the recording electrode is connected to the A-In of the headstage.

or simply via touch, while simultaneously monitoring the response of the ongoing recording; there will be a clear increase in spike number over and above spontaneous activity when stimulating close to or directly on the receptive field. Exclude fibers without a receptive field on the hindpaw, or those fibers with afferent input from the knee/ankle joint, ascertained by physical manipulation of the limbs/joints.

It is important to exclude proprioceptive fibers and afferents from the articulate tissues, as they will have activity not typically associated with neuropathic pain. To ensure that recorded fibers with spontaneous activity are sensory afferents, it is imperative to confirm that they have receptive field(s) on the hindpaw (plantar surface).

Administer drugs/compounds and classify fibers

18. For acute dosing studies, record a period of 20 min of stable baseline activity before administration of the test substance (see Fig. 11.15.8) and a minimum of 40 min of such monitoring post-administration (see Commentary for further protocol options).

19. After completing examination of vehicle/compound effects, insert two 25-G syringe needles, each connected to a 4-mm plug, into the receptive field, to determine the conduction velocity (C.V.) of the recorded filament.

    An alternative method of stimulating responses for C.V. classification can be performed by placing a pair of tungsten stimulation electrodes under a short segment of the sural nerve surgically isolated at the level of the ankle.

20. Connect the two 4-mm plugs to the positive and negative poles of a constant voltage stimulating box, externally triggered by Micro1401 hardware and Spike2 software (see Background Information and Figure 11.15.4, 0.1 Hz, 1 to 50 V, 1 msec pulse width).

    After initiating stimulation of the nerve fiber, evoked response(s) can be identified by their relatively constant latency (i.e., the time between the stimulus artifact and the evoked action potential(s); see Fig. 11.15.5) and, when taking into account the conduction distance, can then be used to determine C.V. The C.V. criteria for the various filament

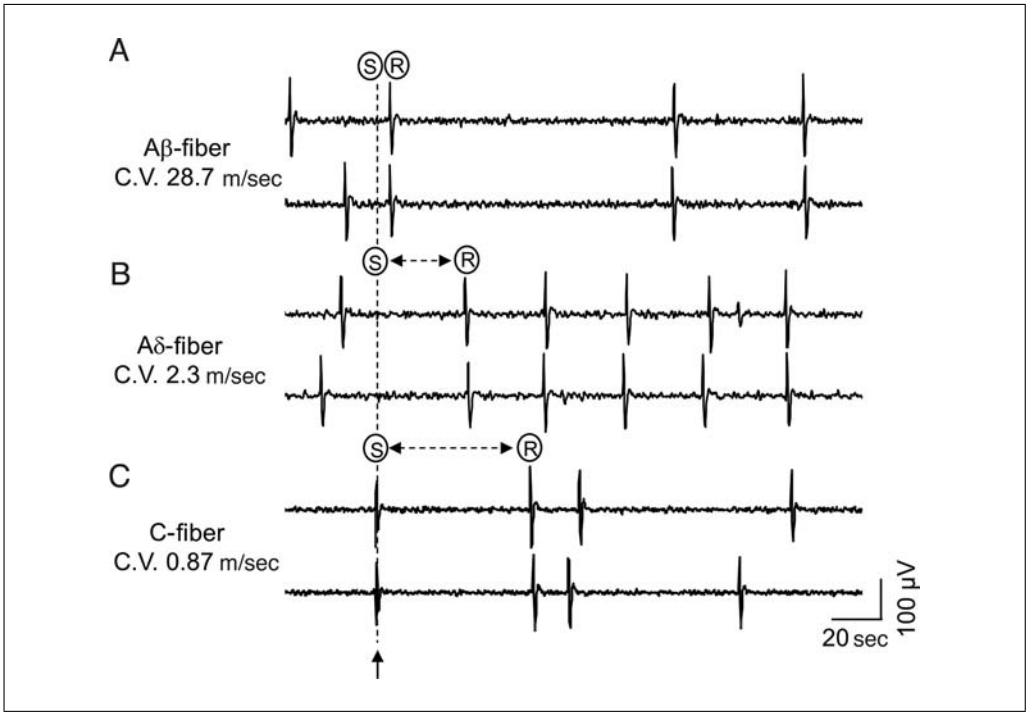

Figure 11.15.5 Examples of classification of recorded fiber type based upon conduction velocity measurements using a standard stimulation-response protocol. **A**, **B**, and **C** show examples of Aβ, Aδ, and C fibers, respectively. The dashed line (marked by ↑) represents the time of stimulation marked in the recording software to allow multiple traces to be aligned post-experiment to aid identification of evoked responses. S: stimulation; R: response.

categories are as follows: C fiber, C.V. ≤ 2 m/sec; A-δ fibers, C.V. > 2 m/sec but ≤ 20 m/sec; A-β fibers, C.V. ≥ 20 m/sec.

Where fibers are spontaneously active, it can be useful to overlay traces to identify the consistent latency of the evoked response. This can be achieved by triggering the software to capture activity at the same time as triggering stimulation of the nerve fiber. Once traces are overlaid, the electrically-evoked response will clearly stand out from ongoing spontaneous action potentials.

21. Terminate the experiment by administering a lethal dose (200 mg/kg, i.v.) of pentobarbital sodium.

22. Save the Spike2 file with all the recorded activity for later offline analysis of the spontaneous and electrically-evoked single fiber activity (see Anticipated Results).

MEASUREMENT OF SPONTANEOUS, WIND-UP, AND AFTER-DISCHARGE ACTIVITY OF WIDE-DYNAMIC-RANGE DORSAL HORN NEURONS, RECORDED FROM THE SPINAL CORD OF NEUROPATHIC MODEL RATS

BASIC PROTOCOL 2

Described in this protocol are basic procedures for monitoring the activity of wide dynamic range (WDR) neurons recorded from the dorsal horn of the spinal cord in the lumbar region (L3-L6) of neuropathic pain–model anaesthetized rats (see Fig. 11.15.6). These procedures are applicable in many rodent models of neuropathic pain after behavioral validation of the pain state (*UNIT 5.32*, see Commentary). Specifically, this protocol describes procedures for monitoring not only the spontaneous activity of these neurons, but also wind-up and after-discharge activity induced by electrical stimulation of the receptive field with a train of pulses (see Fig. 11.15.7). There are also alternate protocols that describe modifications to these procedures to allow investigation of the relative contribution of different components of the nervous system to responses seen. As these experiments are performed on anaesthetized animals, it is important to monitor and

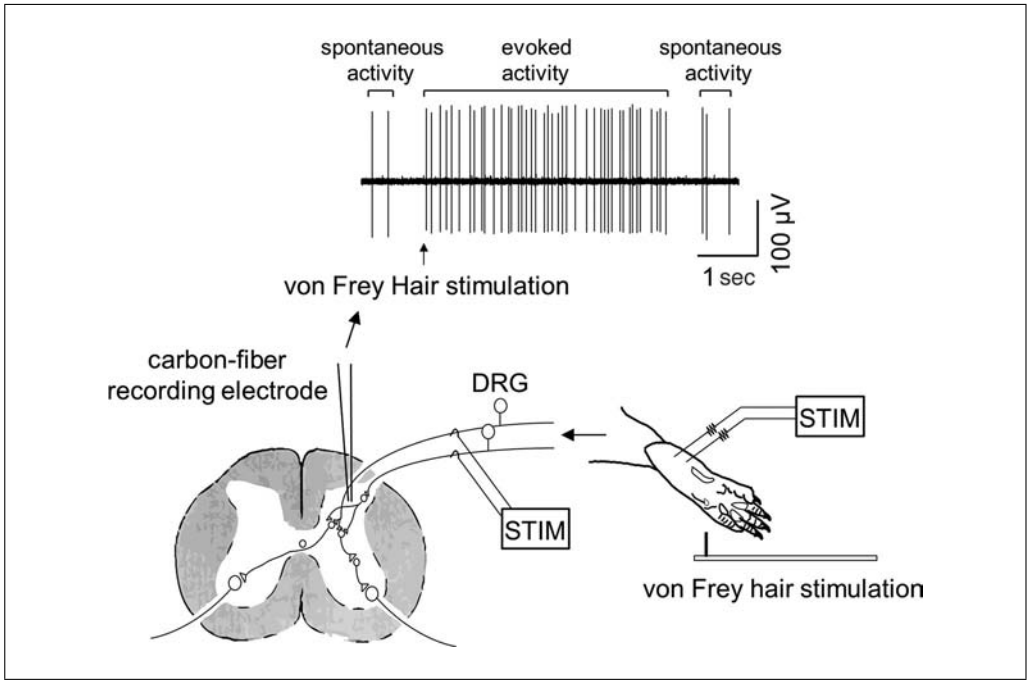

Figure 11.15.6 Schematic diagram showing the setup for recording from a WDR neuron located within the dorsal horn of the spinal cord and the response to mechanical (von Frey hair) stimulation or electrical stimulation applied to the receptive field of the hindpaw plantar surface of a neuropathic pain model rat. As shown, electrical stimulation can also be delivered directly to the dorsal roots.

maintain an appropriate depth of anesthesia and to ensure that blood pressure, heart rate, and body temperature are within the normal physiological range.

Materials

Test substances, e.g., gabapentin, MK801, or morphine (see recipes)
Vibration isolation table with Faraday cage (TMC, Series 20)
Stereotaxic frame with two metal spinal cord clamp sets (ST-7; Narishige)
Single-barrel carbon-fiber microelectrode (Carbostar-1, Kation Scientific)
Hydraulic single-axis micromanipulator (MHW-4, Narishige)

Additional reagents and equipment for monitoring ectopic discharge recorded from nerve filaments of anesthetized rats (Basic Protocol 1)

Prepare for surgery

1. Perform steps 1 to 4 of Basic Protocol 1.

Place animal in the stereotaxic frame

2. Locate the stereotaxic frame on the vibration isolation table close to the edge near the operator and perpendicular to the operator with the head end facing left. Locate the heated blanket within the stereotaxic frame and place the animal prone between the ear bars. Grasp the rat's head, neck, and upper body and guide the fixed right ear bar into the auditory meatus, moving up and down with gentle pressure until accurate placement is confirmed by a popping sound indicative of tympanic membrane rupture. While maintaining the rat's position with the head as horizontal as possible between the ears, bring the left ear bar into position according to the same procedure used for fixing the right ear. Once both ear bars are secured in position, center the animal in the frame, place the upper incisors over the incisor bar, retract the bar until tight, and secure the nose clamp.
3. Perform step 5 of Basic Protocol 1.

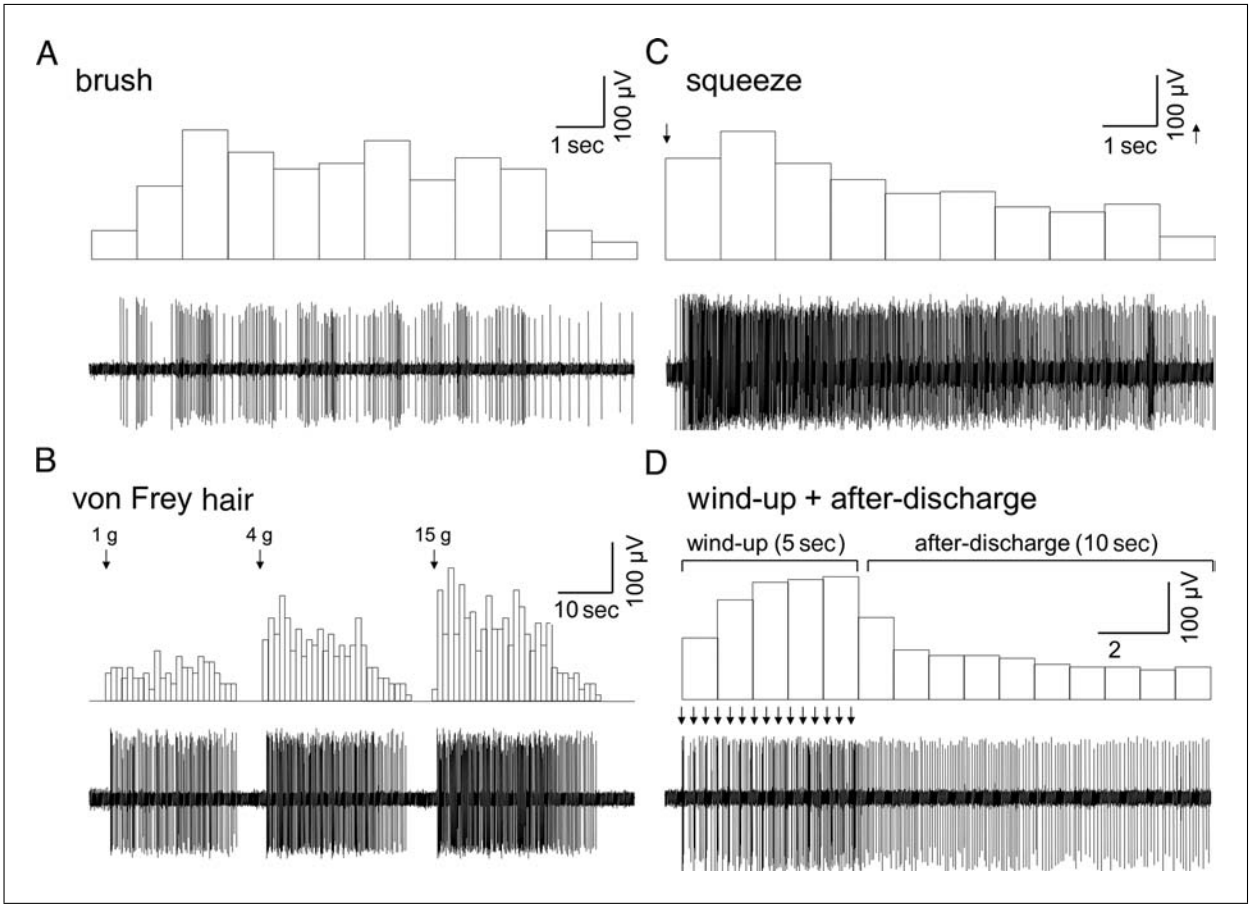

Figure 11.15.7 An example of a typical WDR neuron and its responses to mechanical and electrical stimulation delivered to its receptive field on the hindpaw plantar surface. Mechanical stimulation of increasing intensity delivered via brush (**A**), von Frey hairs of increasing force (1 g, 4 g, 15 g, **B**), and arterial clamp (**C**). Note the increased frequency of action potential firing in line with increasing force of stimulation. (**D**) Wind-up and after-discharge evoked by a train of sixteen electrical pulses (↓) over 5 sec at an intensity of 2× the threshold for C fiber stimulation.

Perform laminectomy

4. Shave the back of the animal covering the caudal thoracic and lumbar region of the spinal cord in the regions T12-L6. Make a paramedial incision through the skin and remove superficial connective tissue.

5. Carry out a laminectomy, removing all lamina bone tissue between T12 and L6 to access lumbar segments L3-L6 of the spinal cord, carefully avoiding damage to the dorsal horn.

   Care should be taken to ensure the laminectomy is correctly located to allow the identification of dorsal horn neurons with their receptive field on the hindpaw. The spinal cord segment containing these neurons will actually be located close to the region where the dorsal roots enter the cord, which is usually 2 to 3 segments rostral (~5 mm) to the equivalent vertebrae segment.

   It may take a few experiments to precisely map the location of dorsal horn neurons whose receptive field is located within the hindpaw, with respect to the overlying vertebral bone segments.

6. Fix the spine with two metal spinal cord clamps, one rostral and one caudal to the exposed spinal cord.

   A clear exposure and secure fixing of the spinal cord will greatly benefit dorsal horn recording stability. However, care must be taken to ensure that the spinal cord is not

stretched during this process, causing traumatic damage and killing neurons, significantly reducing the chance of successfully identifying WDR neurons.

7. Form an oil pool by stitching the edges of the incised skin on the back to a metal frame and cover the spinal cord and surrounding area with warmed (~37°C) mineral oil, to prevent the tissue from drying out.

8. Remove the dura overlying the dorsal surface of the spinal cord carefully under the stereomicroscope using iris scissors and fine forceps.

9. Fix the left hind paw to a metal platform with superglue, with the dorsal surface facing downward, in a natural functional position leaving the paw plantar surface exposed for receptive field exploration and stimulation.

   Fixing the paw to a platform restricts the movement of the ankle joint to avoid movement-induced activity (from proprioceptive cells) when examining the receptive field of selected neurons with either a light brush or von Frey hairs (4 g).

Dorsal horn neuron recording

10. Follow step 15 from Basic Protocol 1.

11. Descend the carbon fiber recording electrode through the surface of the dorsal horn within the L3-L6 region of the spinal cord using a hydraulic single-axis micromanipulator.

12. Begin searching for dorsal horn neurons using low intensity stimulation of the hindpaw (light stroking) as a search stimulus and by lowering the recording electrode in 3- to 5-μm increments until a neuron with receptive field input has been identified. If no neurons are encountered up to a maximum depth of 750 μm, withdraw the electrode from the spinal cord, reposition to another site, and repeat.

13. Once a neuron has been identified and the amplitude of the recording optimized by small adjustments of the position of the recording electrode, refine the location of the receptive field using more focal mechanical stimulation of the hindpaw. Then, identify whether this is a WDR neuron by testing for enhanced responses to increasing mechanical stimulation using three graduated von Frey hairs (1, 4 and 15 g), and to pinching using an arterial clamp. Apply each hair 10 times using a 1-sec-on 1-sec-off protocol. See Figure 11.15.7A,B,C.

Induce wind-up of dorsal horn WDR neurons

14. Insert two 25-G syringe needles, each connected to a 4-mm plug, into the receptive field on the hindpaw.

15. Connect the two 4-mm plugs to the positive and negative poles of a constant voltage-stimulating box, externally triggered by Micro1401 hardware and Spike2 software (see Background Information and Fig. 11.15.4).

16. Use Spike2 software to trigger low-frequency stimulation (0.1 Hz, 1 to 50 V, 1-msec pulse width) of the receptive field to identify electrically evoked action potential(s). Using the latency of the evoked response and the approximate conduction distance, classify the responses based on fiber type (see Basic Protocol 1, annotation to step 20). Subsequently, determine the stimulation intensity threshold required to evoke C-fiber-mediated responses in the recorded neuron.

   If the neuron being recorded does not have an unmyelinated, slowly conducting C-fiber-mediated response/input, then wind-up should not be examined and the search for a WDR neuron must begin again. However, the experimenter may still wish to examine the effects of test compounds on the spontaneous and mechanically evoked activity of this neuron subtype.

17. Induce wind-up and the associated after-discharge by applying a train of 16 pulses over 5 sec at an intensity of 2× threshold (see Fig. 11.15.7 section D and 9). Repeat every 10 min until responses are stable.

18. Record a stable baseline of 20 min before the administration of test substances and a minimum of 40 min post-administration (see Commentary for further protocol options).

19. Follow steps 21 and 22 from Basic Protocol 1.

RECORDING ECTOPIC DISCHARGE FROM MODIFIED PREPARATIONS OF NEUROPATHIC ANIMALS

ALTERNATE PROTOCOL 1

The spontaneous activity or ectopic discharge recorded from fine nerve bundles of neuropathic model rats has a number of different potential origins—e.g., the neuroma (site of injury), axons near the receptive field, the dorsal roots, and/or the spinal cord. Therefore, recording from different levels of the neuraxis allows assessment of the contribution of any given site to the recorded discharge and also identification of the site(s) of action when examining the effects of test substances on this activity (see Fig. 11.15.2). The alternate protocols below detail modification of the procedures to Basic Protocol 1 to facilitate these optional investigations.

Additional Materials *(also see Basic Protocols 1 and 2)*

Stereotaxic frame with two spinal cord clamps (ST-7; Narishige)
Acetate film strip (cut to approx. 1.5 × 20 cm)

To record ectopic discharge of dorsal root ganglia origin

1a. Perform steps 1 to 11 of Basic Protocol 1.

2a. Section the sciatic nerve as distally as possible, while still proximal to the neuroma (section i in Fig. 11.15.2B), and place the proximal-sectioned end (still connected to the DRG and spinal cord; site 3 in Fig. 11.15.2B) onto an opaque plastic platform.

Recording of DRG-generated activity can also be achieved by sectioning either the dorsal roots proximally, i.e., section iii in Figure 11.15.2B, or the sciatic nerve proximal to the ligated area, i.e., section i in Figure 11.15.2B, and teasing and recording fibers from site 2 in Figure 11.15.2B. An oil pool to prevent drying of tissue can be formed by clamping an acetate film onto the skin or by stitching skin flaps onto a metal frame, as described in Basic Protocol 2, step 7.

3a. Follow steps 13 to 16 from Basic Protocol 1.

It is not possible to examine the receptive field in this configuration, as the recording is proximal to the sectioned end of the sciatic nerve. Additionally, although classification of fiber categories is possible, either by placing a pair of stimulation electrodes on dorsal roots L4 and L5 after a laminectomy or more proximally along the sciatic nerve, this procedure is difficult and not recommended, as the conduction distance is very short.

4a. Follow steps 18, 21, and 22 from Basic Protocol 1.

To record ectopic discharge of purely neuroma origin

1b. Follow steps 1 to 8 from Basic Protocol 1, and then remove the surface muscle overlying the sural nerve projection (around the posterior border of the hamstring muscles) and make a small opening around the sciatic nerve projection, proximal to the ligation area.

2b. Section the sciatic nerve as proximally as possible and the sural nerve as distally as possible to the ligation area, i.e., sections i and ii in Figure 11.15.2B, and place

either the proximal-originating sectioned end, i.e., site 1 in Figure 11.15.2A, or the distal-originating section end, i.e., site 4 in Figure 11.15.2B, onto an opaque plastic platform.

Sectioning of the sciatic and sural nerves totally isolates the ligation area (neuroma), ensuring that all activity is of purely neuroma (and surrounding area) origin.

3b. Follow steps 13 to 16 of Basic Protocol 1.

It is not possible to examine the receptive field in this configuration, as the recording is proximal to the sectioned end of the sural nerve.

4b. Follow step 18 from Basic Protocol 1.

5b. Follow steps 20 to 22 from Basic Protocol 1.

To record ectopic discharge from dorsal root filaments

1c. Follow steps 1 to 6 from Basic Protocol 1.

2c. Follow steps 2 to 9 from Basic Protocol 2.

3c. Section the L4 or L5 dorsal root at the point at which it joins the spinal cord, i.e., section iii in Figure 11.15.2B, and place the proximal originating sectioned end, i.e., site 2 Figure 11.15.2B, onto an opaque plastic platform.

In CCI model neuropathic rats (UNIT 5.32, see Commentary), both L4 and L5 dorsal roots can be used for fiber teasing. However, for Chung-model neuropathic rats, the L5 dorsal root has been tightly ligated (UNIT 5.32, see Commentary), leaving many fibers dead or degenerated. Therefore, to increase the chance of a successful recording, teasing L4 is recommended, as there will be many sensitized fibers with spontaneous activity.

4c. Follow steps 14 to 22 from Basic Protocol 1.

To record ectopic discharge or peripheral origin

1d. Follow steps 1 to 8 from Basic Protocol 1, then remove the surface muscle overlying the sural nerve projection (around the posterior border of the hamstring muscles), and make a small opening around the sciatic nerve projection, proximal to the ligation area.

2d. Section the sural nerve distal as proximal as possible while still distal to the nerve injury, i.e., section ii in Figure 11.15.2B, and place the distal-sectioned end, i.e., closer to the receptive field as per site 5 from Figure 11.15.2B, onto an opaque plastic platform.

3d. Follow steps 14 to 22 from Basic Protocol 1.

ALTERNATE PROTOCOL 2

RECORDING WDR NEURON ACTIVITY FROM MODIFIED PREPARATIONS OF NEUROPATHIC ANIMALS

In studies determining the site(s) and mechanism(s) of action of a test substance, it is important to be able to ascertain the contributions of different levels of the neuraxis to the observed effect. When examining the effect of test substances on the activity of WDR dorsal horn neurons, it is possible to exclude any contribution to these effects from either higher levels of the central nervous system or from peripheral aspects, using surgical intervention. In practice this is achieved by determining any change in response after either sectioning the ipsilateral dorsal roots (L3-L6), thereby excluding any peripheral effects on primary afferent drive, or spinalizing the animal at the thoracic (T2 segment) level, thereby excluding any effects from higher centers on descending inhibitory pathways, or indeed after performing both.

For materials, see Basic Protocols 1 and 2.

To record WDR dorsal horn neurons after spinalization

1a. Follow steps 1 to 3 from Basic Protocol 2.

2a. Shave the back of the animal covering the thoracic and lumbar region of the spinal cord in the T2 region and between regions L2 and L6. Make paramedial incisions through the skin in both regions, removing any superficial connective tissue.

3a. Carry out a thoracic laminectomy, removing all lamina bone tissue to access thoracic segment T2.

4a. Using a stereomicroscope, carefully section the entire spinal cord using fine forceps and iris scissors, applying small sections (~2 × 2 mm) of hemostatic sponge or cotton wool soaked in NS to stop any bleeding that may occur.

5a. Perform steps 5 to 19 of Basic Protocol 2.

WDR neuron spontaneous, wind-up and after-discharge activity is very similar to the non-spinalized preparation, although removal of the descending inhibitory pathway may result in higher frequency spontaneous activity and greater wind-up and after-discharge. Good hemostasis is recommended, as excessive bleeding may lower blood pressure leading to unstable or abnormal neural activity.

To record dorsal horn neurons after sectioning the ipsilateral dorsal roots

1b. Follow steps 1 to 8 from Basic Protocol 2.

2b. Using the stereomicroscope, carefully identify and section dorsal roots L3 to L6 as distally as possible using fine forceps and iris scissors, applying small sections (~2 × 2 mm) of hemostatic sponge or cotton wool soaked in NS to stop any bleeding that may occur.

It is not possible to examine the receptive field in this configuration, as the recording is proximal to the sectioned dorsal roots; therefore, classification of the neurons as WDR is unavailable. However, you can still stimulate afferent inputs (to induce wind-up and after-discharge) by stimulating the sectioned dorsal roots.

3b. Place two tungsten stimulation electrodes under one or more of the proximal originating dorsal roots as close as possible to the section site (see Fig. 11.15.6).

4b. Follow steps 10 and 11 from Basic Protocol 2.

5b. Begin searching for spontaneously active dorsal horn neurons by lowering the recording electrode in 3- to 5-μm increments up to a maximum depth of 750 μm. If no neurons are encountered during the full penetration, withdraw the electrode from the spinal cord reposition to another site and repeat.

6b. Follow steps 15 to 19 from Basic Protocol 2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes.

Gabapentin

Dissolve gabapentin (Sigma, cat. no. G154) at a concentration of 100 mg/ml in normal saline (see recipe). Prepare fresh daily.

Lidocaine

Dissolve lidocaine hydrochloride monohydrate (Sigma, cat. no. L5647) at a concentration of 10 mg/ml in normal saline (see recipe). Prepare fresh daily.

MK801

Dissolve MK801 (Sigma, cat. no. M107) at a concentration of 3 mg/ml in normal saline (see recipe). Prepare fresh daily.

Mexiletine

Dissolve mexiletine (Sigma, cat. no. M2727) at a concentration of 5 mg/ml in normal saline (see recipe). Prepare fresh daily.

Morphine

Dilute morphine sulfate injection (Martindale Pharmaceuticals, 10 mg/ml) to a concentration of 3 mg/ml in normal saline (see recipe). Prepare fresh daily.

Normal saline

0.9% (w/v) NaCl: 9 g of NaCl per liter water. Store up to 2 to 3 months at 4°C.

Normal saline (NS), heparinized

Dissolve heparin sodium salt (from porcine intestinal mucosa; Sigma, cat. no. H4784) at a concentration of 50 U/ml in normal saline (see recipe). Store up to 2 to 3 months at 4°C.

Pentobarbital sodium

Dissolve pentobarbital sodium (Sigma, cat. no. P3761) at a concentration of 200 mg/ml in normal saline (see recipe). Store up to 2 to 3 months at 4°C.

NOTE: *Pentobarbital sodium is a controlled substance, and as such the use of this material must follow officially approved procedures under necessary licensing regulations.*

Urethane, 12% (w/v)

Dissolve urethane (Sigma, cat. no. U2500) at a concentration of 12 g/100 ml in normal saline (see recipe) at least one day before use. Store up to 1 month at 4°C.

COMMENTARY

Background Information

Wall and Gutnick (1974) first reported the phenomenon of spontaneous activity, or ectopia, in the axons of animals receiving a nerve section or axotomy. Ectopic discharge has also been shown to be a prominent feature in other surgically induced animal neuropathic pain models, as revealed by electrophysiological methods in the CCI model (Zhao et al., 2007) and Chung model (Hopkins et al., 2013). Furthermore, the ectopic discharge generated in rodent models is closely related to the spontaneous, ongoing pain in neuropathic patients (Campbell et al., 1988), demonstrating the critical involvement of this activity in the sensation of pain. This evidence also supports the application of these animal models in predicting the efficacy of novel therapeutic agents in man (Zhao et al., 2007). Similarly, WDR neurons in the dorsal horn of the spinal cord play a pivotal role in the processing of pain information. Repeated stimulation of the receptive field of WDR neurons leads to the spatial and temporal summation of synaptic transmission in the dorsal horn, and wind-up and after-discharge are two measures of this outcome. Although this differs from the sensitization induced by persistent stimulation from a nerve injury or inflammation, the underlying molecular mechanisms are thought to be shared (Baranauskas and Nistri, 1998; Herrero et al., 2000). As this involves many aspects of neuronal circuitry in the spinal cord, with numerous mechanistic processes, any drug intervention that addresses a component of this (e.g., glutamate: Budai and Larson, 1998; Kovacs et al., 2004; You et al., 2004; or neuropeptides: Coste et al., 2008) will be able to manipulate signal processing and change the functional output. In a neuropathic state, the spinal sensitization is characterized by an increase in spontaneous activity and enhanced responsiveness of WDR neurons to mechanical and electrical stimulation (Palecek et al., 2004). This is associated with up-regulation of spinal cord receptors, e.g., cannabinoid receptors, with substantial evidence showing the efficacy of drugs in a neuropathic pain state to

significantly differ from that found in naïve animals (Svendsen et al., 1999; Suzuki and Dickenson, 2006). It is important to remember that the phenomena of pain sensitization and the methods for monitoring this are not limited to neuropathic pain models, and also occur in other disease models, such as models of inflammatory pain (Svendsen et al., 1999; Nackley et al., 2004).

Limitations and alternatives

When investigating the ability of novel test substances to alleviate neuropathic pain, it is typical to determine if there is any reduction in pain behavior in one or many of the various neuropathic models available, using behavioral readouts such as paw withdrawal threshold. These whole-animal experiments directly relate the effect of the substance to the presentation of pain behavior but, importantly, do not allow investigation into the site of action of the substance, effects on specific fiber types, or effects on functional neuronal phenomena, e.g., wind-up. The ability to investigate these aspects of test substances are among the critical advantages in performing the experiments described in this unit. The procedures described in this protocol also allow investigation into the processes involved in the development of neuropathy, and can provide important information on associated neurophysiological changes. However, they do not provide direct evidence of mechanism at the intracellular or molecular level. For higher resolution of pathophysiological and pharmacological mechanism, electrophysiological studies using patch-clamp recording techniques from spinal cord slice and acutely dissociated DRG preparations, in combination with molecular biology and fluorescent imaging, are recommended after in vivo electrophysiology. Additionally, other in vitro electrophysiological investigations such as the monitoring of compound action potential activity from isolated sciatic nerve-DRG-dorsal root preparations can also be applicable in determining the mechanism of test substances on, for example, peripherally restricted ion channels. Importantly, these ex vivo preparations are all capable of being isolated from both naïve and neuropathic pain model rats. As mentioned, the techniques described in this protocol are also applicable in other species, including the mouse. However, with respect to the measurement of conduction velocity in single-fiber recordings, consideration must be given to the shorter distances between the site of stimulation and recording in the mouse, as this will give rise to difficulty in fiber categorization. This is also relevant in Alternate Protocol 1, where care must be taken to ensure that the conduction distance over which velocity is examined is as great as possible. With respect to Alternate Protocol 2, the recording of dorsal horn neurons in a dorsal root–sectioned animal, a clear limitation of this technique is the inability to characterize spontaneously active neurons as being WDR, due to the connection between the neuron and the peripheral receptive field having been severed. However, it is still worthwhile assessing the spontaneous, wind-up, and after-discharge activity of these neurons. It is also worth mentioning that besides the phenomenon of wind-up, there are other methods for inducing plasticity in WDR neurons using high-frequency stimulation to generate long-term potentiation in evoked responses (Baranauskas and Nistri, 1998). Finally, although clear examples of test compounds affecting the measured output from these two protocols are given (see Figs. 11.15.8 and 11.15.9), it is notable that there are plenty of alternative compounds to use as reference or positive control, both to demonstrate proof of concept and to allow comparison with the effects of novel test substances. Careful selection of an appropriate reference substance might be an important consideration when targeting particular mechanisms, i.e., using MK801, a selective NMDA receptor antagonist, if investigating glutamate receptor function.

Critical Parameters and Troubleshooting

It must be remembered that the procedures described in this unit are carried out in living animals, and that there is a significant biological variation between individuals. This is highlighted by the use of anesthetic in these experiments, which must be administered according to the response of the animal and the stability of measured physiological variables, rather than by a prescribed amount. There can also be some variability in the precise anatomy between animals, e.g., in the exact location of peripheral nerves and/or the position of the L4-L6 dorsal roots relative to their associated L4-L6 spinal segments. Generally, the most significant component in achieving consistent and reproducible experiments is the stable condition of the animal. Taking as much care as possible with the anesthetic induction and maintenance, the surgical preparation of the animal, and in particular the handling of the nervous tissues, will allow the greatest chance of success. In addition, as these experiments involve the recording of very small

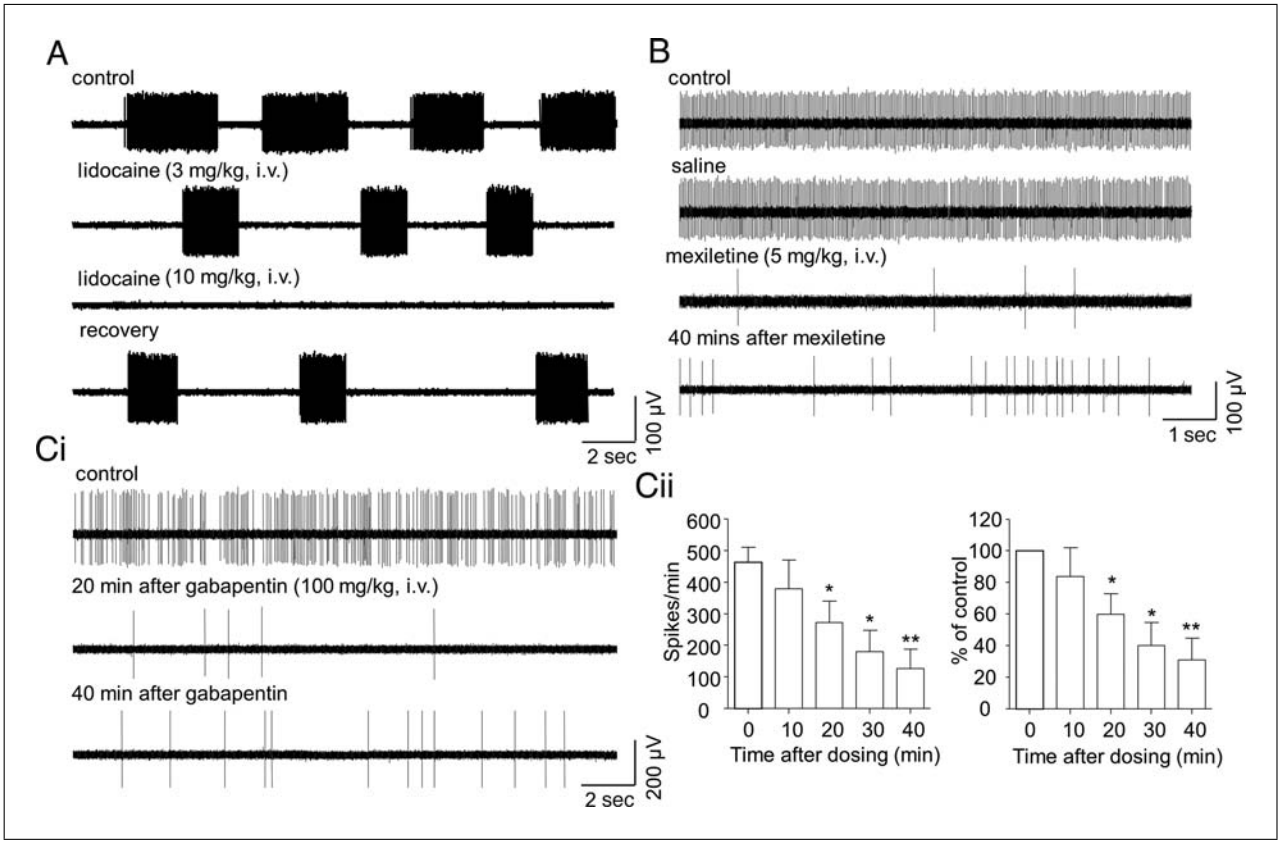

Figure 11.15.8 Representative examples of compounds tested on ectopic discharge in neuropathic pain model rats. (**A**) Burst-like ectopic discharge recorded from a sural nerve filament (site 4 in Fig. 11.15.2B) of a CCI model rat was dose-dependently inhibited by i.v. injection of lidocaine. (**B**) Ectopic discharge recorded from a sciatic nerve fiber (site 1 in Fig. 11.15.2A) was blocked by i.v. injection of mexiletine, a sodium channel blocker. (**Ci**) ectopic discharge recorded from a dorsal root filament (site 2 in Fig. 11.15.2B) was blocked by i.v. injection of gabapentin. (**Cii**) pooled data ($n = 8$) showing the inhibition of gabapentin. *, **; $P < 0.05$ and 0.01, compared to control at time 0, paired Student's *t*-test.

amounts of electrical activity, to ensure an optimal signal-to-noise ratio it is essential to regularly check all electrical connections not only between recording and stimulating contacts, but also between all connections to the central earthing point, to minimize interference in your recorded signal from electrical noise.

Neuropathic pain model validation

These experiments are predominantly designed to incorporate investigation of pain-related activity using electrophysiological recording techniques. As such, the quality and reproducibility of the neuropathic pain model at the time the experiment is performed is of vital importance and directly related to the consistency of the electrophysiological results achieved. It is therefore advisable to carry out behavioral validation of the pain model at regular intervals during the development of the model, and close to the time at which the electrophysiological experiment commences, using standard techniques that are appropriate to the model in use; e.g., in the CCI model, animals must demonstrate mechanical allodynia ipsilateral to the site of recording.

Test substance examination

For the experiments described in this unit, selection of the administration method and pre-treatment time of test substances before commencing experiments is also critical to ensure sufficient penetration of the relevant tissue (e.g., the spinal cord) and a maximal chance of successfully examining efficacy. Therefore, information on the pharmacokinetic properties of the compound being tested is essential to guide these elements of study design. Most methods of administration (i.v., i.p., i.m., s.c., p.o.) can be applied in the performance of the described procedures with minor modification, although there are limitations to some—e.g., oral gavage. Although oral gavage is possible when an animal is in the stereotaxic frame, the absorption of compounds through the enteric system is likely to be significantly affected by the reduced gastric motility associated with anesthesia. In addition, some compounds may not penetrate the central nervous system, e.g., peptides, in which case intrathecal

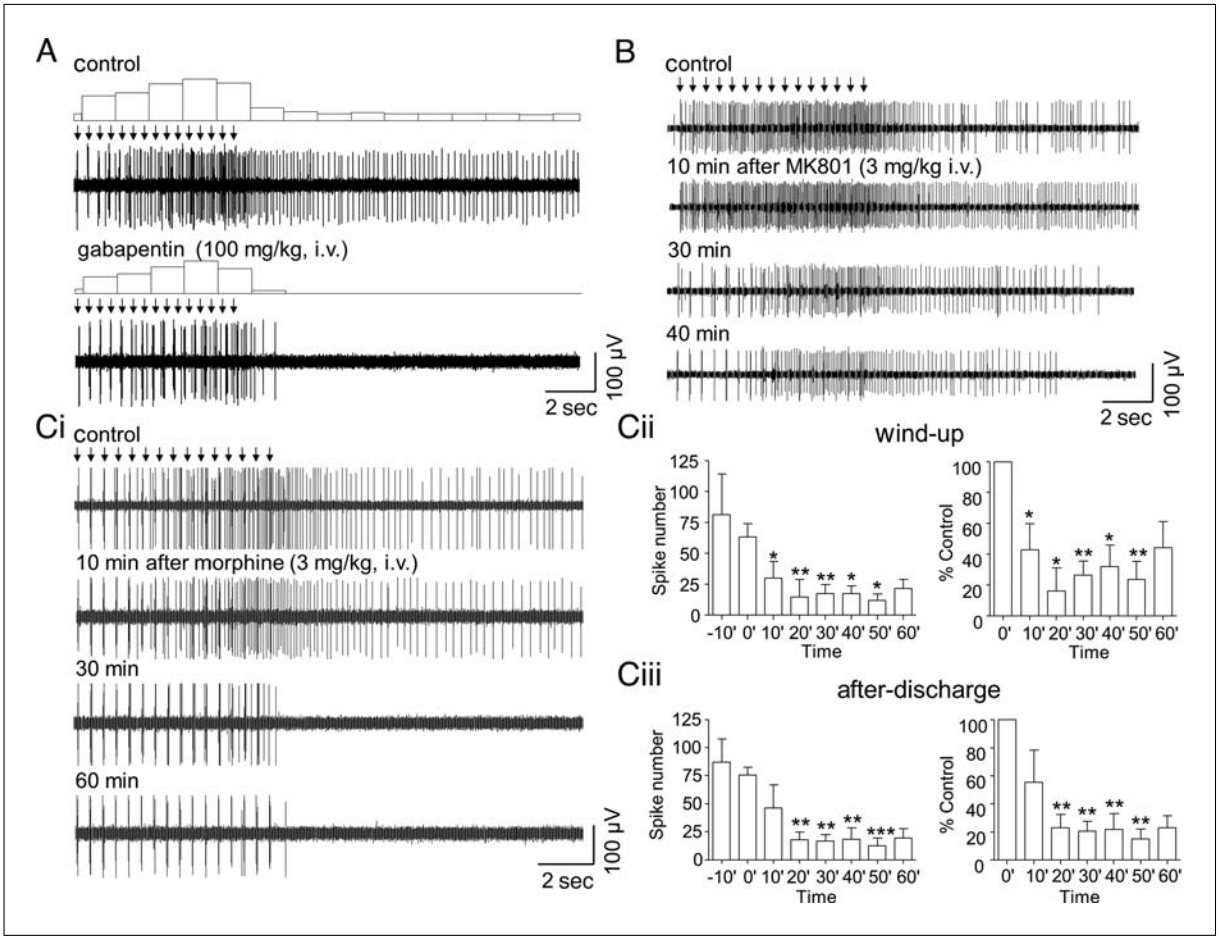

Figure 11.15.9 Representative examples of compounds tested on wind-up and after-discharge in WDR neurons, showing the effects of gabapentin (**A**), the NMDA receptor antagonist MK801 (**B**), and morphine (**C**). **Ci**, raw data; **Cii** and **Ciii**, pooled data ($n = 4$) showing the depression of wind-up and after-discharge induced by morphine administration over time. *, **, ***; $P < 0.05$, 0.01 and 0.001, compared to control at time 0', paired Student's *t*-test.

administration may be necessary. Furthermore, if advance pharmacodynamic information on test compounds is also available, this can also help further tighten the resolution of the study design by directly relating the desired effect of the compound to a known administration route and pre-treatment time, using autoradiography or biochemistry techniques. However, where only PK data is available and an appropriate pre-treatment time is selected on this basis, the procedures described in this unit can actually prove valuable in generating PD data, by directly comparing compound exposure readouts from blood and/or tissue samples taken at the end of recordings with effects seen.

Acute versus chronic dosing

As described in this unit, compounds can be examined for their effects after acute administration, with a rapid onset of the response, e.g., lidocaine and mexiletine (see Fig. 11.15.8); where possible, wash-out or recovery of control activity should always be attempted. In this case, depending on the precise nature of the effects being examined, the authors recommend a group size of eight rats to give sufficient power to make worthwhile statistical comparison between groups. In contrast, it may be desirable to examine the effect of a test substance or treatment after chronic treatment—i.e., over several days or weeks. For chronic administration, a slightly different experiment design is required in order to make adequate comparisons between treatment regimens. For assessing the effect of a treatment on neuropathic ectopic activity, instead of monitoring fibers for up to an hour or more, the aim is to record the firing rate and pattern of as many fibers as possible from each animal (as a guide, we estimate that it is feasible to tease up to 250 fibers from the sural nerve). In this design, we would recommend a group size of six rats; the number of active fibers with spontaneous activity can then be calculated as a percentage of the total number of fibers recorded and compared to the effects of vehicle; effects of treatment on firing patterns can also be interpreted

(Zhao et al., 2007). In this case we would not recommend examining the effects of treatment on conduction velocity due to the time taken to examine this for each fiber, making the experiment unfeasible.

Recording quality

When recording single-fiber or WDR neuron activity for a long period, particularly after a treatment in which the spontaneous ectopic discharge or electrically evoked responses have been completely inhibited, the experimenter might have some concern about whether the unit is still in good health or has died, or that the recording quality has diminished. It is therefore recommended to examine the condition of the recorded unit to remove such doubt. A commonly used method is to apply a von Frey hair, 4 g or 10 g, to the receptive field regularly, e.g., every 10 min. If after application of a von Frey hair of >10 g, the unit is still not responding, it can be concluded either that the unit has died or the quality has completely diminished and the recording should be abandoned. In the WDR neuron experiments, small changes in the position of the recording electrode relative to the recorded neuron are likely as a result of tissue shrinkage or swelling, and this will affect the amplitude of recorded action potentials—i.e., the quality of recording. To maintain the highest-quality recording after 2 hr, this must be compensated for by altering the position of the electrode with small adjustments (1 to 2 μm) in the micromanipulator, although great care must be taken, as any substantial movement could lead to cell penetration, resulting in a complete loss of the recording.

Multi-unit activity

For single-fiber experiments, if the initial recording assessment shows that there are two live units in one bundle and the action-potential amplitude of the two units are significantly different, the recording can be continued, as these two units can be easily discerned and analyzed separately using Spike2 software or manually at a later time (Fig. 11.15.3B). If a bundle contains three or more live units (Fig. 11.15.3C), with many different action potential amplitudes, further teasing of the bundle to separate the active fibers is recommended. However, it must be highlighted that the ideal situation is to record just a single unit (Fig. 11.15.3A), because the longer the recording continues, the greater the chance that the action potential amplitude will change over time, which in the case of a two-unit recording may result in the units becoming indiscernible, producing an ambiguous result. However, in WDR neuron experiments, although, as mentioned, the position of the tip of the recording electrode can be slightly adjusted, the relative spatial distance between adjacent neurons cannot be changed. In a situation when the experimenter feels it would be difficult to discern two (or more) neurons in later offline analysis, it is recommended to withdraw the electrode, reposition it, and identify a further neuron.

Anticipated Results

Basic Protocol 1

The primary observation when recording ectopic activity is the firing rate or frequency of the response (in Hz or spikes/time unit), and the subsequent comparison of this between control and test conditions (Hopkins et al., 2013). This is a relatively easy output to analyze, by simply counting the number of spikes or action potentials over a given time period. This measurement can either be examined over the entire recording period and split into bins representing ongoing activity throughout baseline and the treatment condition (a repeated measures analysis), or maximal/minimal periods during treatment can be selected and compared with baseline and between treatment conditions (a paired analysis). It is important to highlight that the frequency of ectopic discharge can vary quite significantly, with activity 2 weeks following CCI surgery ranging from 18 to 3903 spikes per min (Zhao et al., 2007). In dorsal root fiber recordings from Chung-model rats 1 week after surgery, about 70% of bundles exhibited spontaneous ectopic discharge, with an average frequency of around 600 spikes per min. However, significantly, this frequency diminishes over time (Han et al., 2000), an important consideration when performing these experiments in pain model animals; they have a limited window in which to study the effects of test substances, and this should be maintained consistently between cohorts. Responses can also be grouped according to the pattern of spontaneous activity observed; although it is notable that the pattern of ectopic discharge reported in the literature does vary, a number of patterns of firing including burst-like, regular, and irregular are all commonly seen (see Fig. 11.15.10), and this is largely irrespective of the specific neuropathic model. For example, in CCI-model rats, the proportion of firing patterns observed in a study was: burst-like, 47.8%, regular, 8.5%, paired/tripled, 18.2%, and irregular,

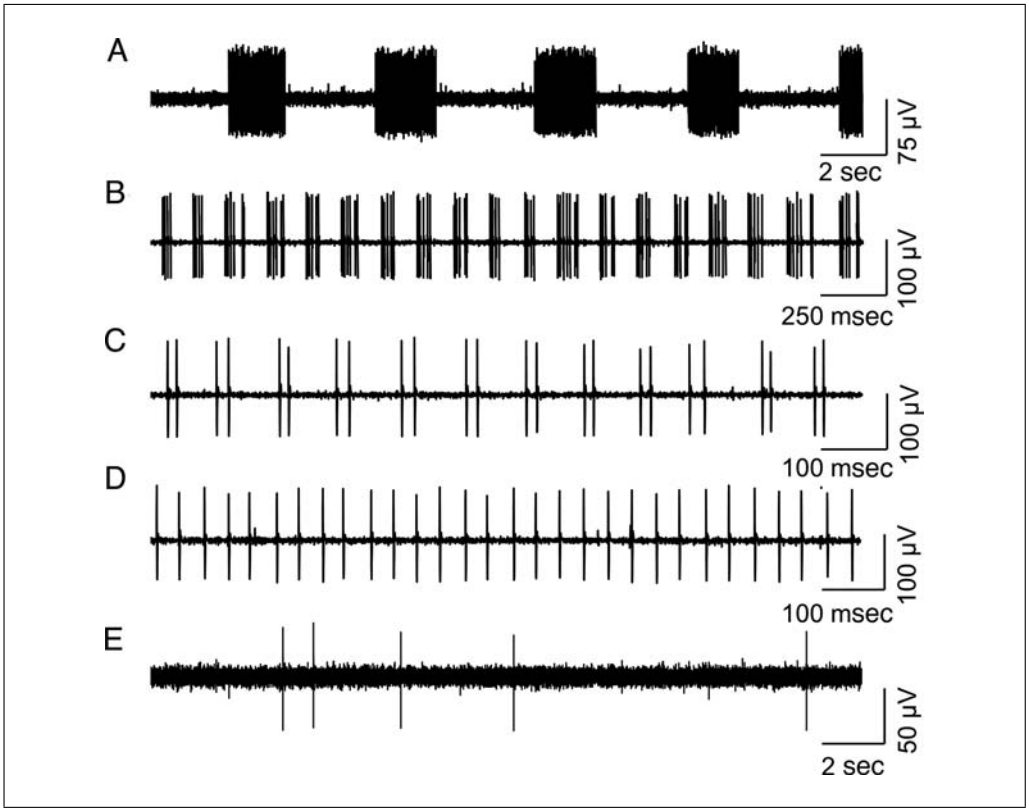

Figure 11.15.10 Differing firing patterns of spontaneous ectopic discharge recorded from sural nerve fibers of CCI model rats. (**A)** An example of typical burst-like firing. (**B**) A further example of burst-like firing exhibiting shorter intervals between bursts, generally referred to as 'fast burst-like' firing. (**C**) An example of 'paired/triplet' firing with discharge occurring in regular patterns of two or three spikes. (**D**) An example of regular firing discharge, with spikes occurring at relatively fixed intervals. (**E**) An example of irregular firing, showing atypical spontaneous activity without a clearly discernible pattern, generally occurring at a low frequency.

25.5% (Zhao et al., 2007). These percentages will vary from study to study and from model to model. Equally, the firing frequency and patterns differ from surgically induced neuropathy and from diabetes- or chemotherapy-induced neuropathy-model animals. In surgically induced neuropathy, the firing frequency is usually higher, ranging from several hundred to several thousand impulses/min, and some typical firing pattern(s) have been characterized (see Fig. 11.15.10). Another method to classify responses is based upon the fiber type of the recorded unitary activity, based on conduction velocity (see Fig. 11.15.5). In contrast to naïve conditions, in neuropathic pain models, spontaneous ectopic discharge is frequently observed in Aβ- and Aδ-fibers with few, if any, active C-fibers (Wall and Gutnick, 1974). Aβ-fibers, in naïve conditions, sense and conduct the tactile and pressure sensations and are normally of low threshold. Interestingly, the functional change in Aβ fibers may be a key drive related to the observation of mechanical, particularly dynamic, allodynia (Devor, 2009). Another consideration in the analysis of ectopic discharge relates to identification of the site of action of a test substance by monitoring spontaneous activity from one of the options listed in Alternate Protocol 1; the origin of the discharge could be from axons, the neuroma itself, or the DRG, and this will obviously impact the effects seen. For example, in the sural nerve of CCI rats, approximately one-third of the observed ectopic discharge is of neuroma origin. In contrast, for diabetes- and chemotherapy-induced neuropathic model animals, there is a greater chance of observing ectopic discharge generated from the peripheral end (close to the receptive field). The firing pattern is also often atypical in these models, with most fibers firing with an irregular pattern, having a lower firing frequency compared to surgically induced neuropathic models, and with a significant percentage of the spontaneous activity being C-fiber mediated (Burchiel et al., 1985). Additionally, for these non-surgically-induced neuropathies, it is easier to categorize the fiber

category (i.e., Aβ, Aδ, or C), as there is less ectopic discharge, allowing easier identification of electrically evoked responses. In surgically induced model rats, this process can be more difficult, as high-frequency spontaneous activity may make distinguishing the actual stimulus-induced response from the frequent spontaneous action potentials difficult. It is therefore useful to mark the time of stimulation within the recording software using a separate channel to allow post-experiment identification of the precise time point of stimulation. It is also imperative to repeat the stimulation several times to allow traces to be aligned post-experiment, further confirming the identification of electrically evoked responses and discriminating this from spontaneously occurring action potentials (see Fig. 11.15.5).

Basic Protocol 2

Spontaneous activity, wind-up, and after-discharge can be all measured from the same animal in a single experiment (see Fig. 11.15.6). Stimulation of the receptive field for wind-up and subsequent after-discharge measurement occurs generally only at an interval of 5 to 10 min, allowing measurement of spontaneous firing frequency between periods of stimulation (approximately 4 min), immediately before and generally up to 120 min after the administration of vehicle or a test compound. Wind-up is measured based on methods described by Svendsen et al. (1999). Briefly, wind-up is calculated as the total number of evoked action potentials in response to 16 electrical pulses minus 16 times the total number of action potentials induced by the first of the sixteen pulses, and can be calculated using the following formula: wind-up = $B - (A \times 16)$, where A is the number of action potentials within 300 msec after the first electrical stimulation and B is the total number of action potentials induced by the whole train of electrical pulses (16 pulses) in that 5 sec. If, in some cases, wind-up is completely inhibited following compound administration, the number of wind-up action potentials (B) may be smaller than control levels ($A \times 16$), leading to a negative readout. In such cases, wind-up can be arbitrarily set as 0 (completely inhibited) for ease of statistical analysis. Finally, after-discharge is calculated as the total number of action potentials recorded within 10 sec, starting 300 msec after the last electrical pulse of the train, i.e., the 16th electrical stimulus (Fig. 11.15.6). It is notable that the number of action potentials associated with spontaneous activity, wind-up, and after-discharge varies widely from neuron to neuron. Therefore, the percentage change relative to control should be used to normalize the data, with the value for spontaneous activity measured immediately before vehicle or drug injection (0 min) used as control (100%). Activity measured after vehicle or drug injection at the various time points should be divided by the number of action potentials at 0 min, and converted to a percentage for statistical analysis.

Compound testing

Basic Protocols 1 and 2 can be used for the investigation of novel compounds in development for the treatment of pain. When performing a study such as this, using either set of techniques, it is necessary to compare the effects of novel compounds with drugs known to have an effect on electrophysiological outputs of these models of neuropathic pain. In addition, it may be optimal to consider the mode of action of the novel compound in order to make the comparison with a reference compound more relevant. Figures 11.15.8 and 11.15.9 illustrate the effects of compounds known to reduce the characteristic 'pain' activity of the nervous system with established clinical efficacy. Morphine (Fig. 11.15.9), an opioid receptor agonist, induces a robust depression of pain that can be clearly demonstrated using electrophysiological recording techniques. Gabapentin (Figs. 11.15.8 and 11.15.9) is one of very few specific treatments for neuropathic pain, and shows good efficacy in depressing both ectopic discharge and spontaneous and wind-up activity in nerve bundles of the peripheral nervous system and WDR neurons of the dorsal horn, respectively. Finally, mexiletine (Fig. 11.15.8B), a sodium channel blocker, has also shown efficacy in the treatment of refractory pain in particular, and is effective in depressing the ectopic discharge characteristic of neuropathic pain models in the periphery.

Time Considerations

As discussed above, the timeline for completing the experiments detailed in these protocols can vary significantly depending on the protocol used and the associated time for surgical preparation; the time spent identifying and stabilizing neural activity; the time course of the treatment effects; and how many doses/concentrations are tested in a single preparation. Although it is feasible to record stable ectopic discharge for up to 2 hr and WDR neuron activity for up to 4 hr, as a guide, recording 60 min of activity following treatment should be achievable without

significant difficulty. Within this time window, the quality of the recording, as assessed by the signal-to-noise ratio and the consistent amplitude of the recorded unit(s), needs to be stable and satisfactory. In addition, the time of recording is not limitless, particularly with regard to the time under anesthesia, which the authors would recommend limiting to 8 hr after induction. In reality, in ectopic discharge or WDR neuron experiments, achieving the necessary stability to monitor the spontaneous and evoked activity of single fibers and neurons for 2 hr or more is difficult, although when performing the population experiments described above, a complete experiment timeframe of up to 6 or 7 hr is realistic and worth bearing in mind if considering these experiments. As a general guide and with practice, the cannulation surgery, placement in the stereotaxic frame, laminectomy, and initial electrode positioning can be done within 90 min. The authors would then recommend a period of 30 to 60 min to allow the animal's conditions to stabilize before identifying neural activity. The subsequent optimization process, e.g., to assess stimulation thresholds for action potential generation and to allow a stabilization period, should take approximately 30 min before commencing baseline recording. Allowing for a 20-min pre-dosing baseline recording period and a 60-min post-dosing period, a total experiment time of between 4 and 5 hr should be anticipated. Finally, it must be highlighted that these are recordings from living animals and there will be significant variation between experiments, including the time taken to identify and optimize responses and the time required for responses to stabilize sufficiently to ensure the best chance of a successful experiment.

Literature Cited

Baranauskas, G. and Nistri, A. 1998. Sensitization of pain pathways in the spinal cord: Cellular mechanisms. *Prog. Neurobiol.* 54:349-365.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. 2009. Cellular and molecular mechanisms of pain. *Cell* 139:267-284.

Bennett, G.J. and Xie, Y.-K. 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.

Budai, D. and Larson, A.A. 1998. The involvement of metabotropic glutamate receptors in sensory transmission in dorsal horn of the rat spinal cord. *Neuroscience* 83:571-580.

Burchiel, K.J., Russell, L.C., Lee, R.P., and Simm, A.A.F. 1985. Spontaneous activity of primary afferent neurons in diabetic BB/Wistar rat: A possible mechanism of chronic diabetic neuropathic pain. *Diabetes* 34:1210-1213.

Campbell, J.N. and Meyer, R.A. 2006. Mechanisms of neuropathic pain. *Neuron* 52:77-92.

Campbell, J.N., Raja, S.N., Meyer, R.A., and Mackinnon, S.E. 1988. Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 32:89-94.

Cohen, S.P. and Mao, J. 2014. Neuropathic pain: Mechanisms and their clinical implications. *BMJ* 348:f7656.

Coste, J., Voisin, D.L., Miraucourt, L.S., Dallel, R., and Luccarini, P. 2008. Dorsal horn NK1-experessing neurons control windup of downstream trigeminal nociceptive neurons. *Pain* 137:340-351.

Devor, M. 2009. Ectopic discharge in Aβ afferents as a source of neuropathic pain. *Exp. Brain Res.* 196:115-128.

Djouhri, L., Fang, X., Koutsikou, S., and Lawson, S.N. 2012. Partial nerve injury induces electrophysiological changes in conducting (uninjured) nociceptive and non-nociceptive DRG neurons: Possible relationships to aspects of peripheral neuropathic pain and paresthesia's. *Pain* 153:1824-1836.

Donovan, J. and Brown, P. 2006. Parenteral injections. *Curr. Protoc. Immunol*. 73:1.6.1-1.6.10.

Han, H.C., Lee, D.H., and Chung, J.M. 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. *Pain* 84:253-61.

Herrero, J.F., Laird, J.M.A., and Lopez-Garcia, J.A. 2000. Wind-up of spinal cord neurons and pain sensation: Much ado about something? *Prog. Neurobiol.* 61:169-203.

Hopkins, S.C., Zhao, F.Y., Bowen, C.A., Fang, X., Wei, H., Heffernan, M.L., Spear, K.L., Spanswick, D.C., Varney, M.A., and Large, T.H. 2013. Pharmacodynamic effects of a D-amino acid oxidase inhibitor indicate a spinal site of action in rat models of neuropathic pain. *J. Pharmacol. Exp. Ther.* 345:502-511.

Khan, G.M., Chen, S.-R., and Pan, H.L. 2002. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. *Neuroscience* 114:291-299.

Kim, S.H. and Chung, J.M. 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50:355-363.

Kovacs, G., Kocsis, P., Tarnawa, I., Horvath, G., Szombathelyi, Z., and Farkas, S. 2004. NR2B containing NMDA receptor dependent windup of single spinal neurons. *Neuropharmacology* 46:23-30.

Latremoliere, A. and Woolf, C.J. 2009. Central sensitization: A generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10:895-926.

Leadley, R.M., Armstrong, N., Lee, Y.C., Allen, A., and Kleijnen, J. 2012. Chronic diseases in the European Union: The prevalence and health

cost implications of chronic pain. *J. Pain Palliat. Care Pharmacother.* 26:310-325.

Mendell, L.M. 1966. Physiological properties of unmyelinated fiber projection to the spinal cord. *Exp. Neurol.* 16:316-332.

Nackley, A.G., Zvonok, A.M., Makriyannis, A., and Hohmann, A.G. 2004. Activation of cannabinoid CB2 receptors suppresses C-fiber responses and windup in spinal wide dynamic range neurons in the absence and presence of inflammation. *J. Neurophysiol.* 92:3562-3574.

Palecek, J., Neugebauer, V., Carlton, S.M., Iyengar, S., and Willis, W.D. 2004. The effect of a kainite GluR5 receptor antagonist on responses of spinothlamic neurons in a model of peripheral neuropathy in primates. *Pain* 111:151-161.

Schaible, H.G. 2007. Peripheral and central mechanisms of pain generation. *Handb. Exp. Pharmacol.* 177:3-28.

Seltzer, Z., Dubner, R., and Shir, Y. 1990. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43:205-218.

Suzuki, R. and Dickenson, A.H. 2006. Differential pharmacological modulation of the spontaneous stimulus-independent activity in the rat spinal cord following peripheral nerve injury. *Exp. Neurol.* 198:72-80.

Svendsen, F., Rygh, L.J., Hole, K., and Tjølsen, A. 1999. Dorsal horn NMDA receptor function is changed after peripheral inflammation. *Pain* 83:517-523.

Wall, P.D. and Gutnick, M. 1974. Ongoing activity in peripheral nerves: The physiology and pharmacology of impulses originating from a neuroma. *Exp. Neurol.* 43:580-593.

Woolf, C.J. 2011. Central sensitization: Implications for the diagnosis and treatment of pain. *Pain* 152:S2-S15.

Xiao, W.H., Zheng, H., and Bennett, G.J. 2012. Characterization of oxaliplatin-induced chronic painful peripheral neuropathy in the rat and comparison with the neuropathy induced by paclitaxel. *Neuroscience* 203:194-206.

You, H.J., Morch, C.D., and Arendt-Nielsen, L. 2004. Electrophysiological characterization of facilitated spinal withdrawal reflex to repetitive electrical stimuli and its modulation by central glutamate receptor in spinal anaethetised rats. *Brain Res.* 1009:110-119.

Zhao, F.Y., Spanswick, D., Martindale, J.C., Reeve, A.J., and Chessell, I.P. 2007. GW406381, a novel COX-2 inhibitor, attenuates spontaneous ectopic discharge in sural nerves of rats following chronic constriction injury. *Pain* 128:78-87.

Zhu, Y.F., Wu, Q., and Henry, J.L. 2012. Changes in functional properties of A-type but not C-type sensory neurons in vivo in a rat model of peripheral neuropathy. *J. Pain Res.* 5:175-192.