

# Latent Sensitization: A Model for Stress-Sensitive Chronic Pain

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Latent sensitization is a rodent model of chronic pain that reproduces both its episodic nature and its sensitivity to stress. It is triggered by a wide variety of injuries ranging from injection of inflammatory agents to nerve damage. It follows a characteristic time course in which a hyperalgesic phase is followed by a phase of remission. The hyperalgesic phase lasts between a few days to several months, depending on the triggering injury. Injection of  $\mu$ -opioid receptor inverse agonists (e.g., naloxone or naltrexone) during the remission phase induces reinstatement of hyperalgesia. This indicates that the remission phase does not represent a return to the normal state, but rather an altered state in which hyperalgesia is masked by constitutive activity of opioid receptors. Importantly, stress also triggers reinstatement. Here we describe in detail procedures for inducing and following latent sensitization in its different phases in rats and mice. © 2015 by John Wiley & Sons, Inc.

Keywords: chronic pain • constitutive activity • hyperalgesia • latent sensitization • neuropathic pain • opioid receptor • stress

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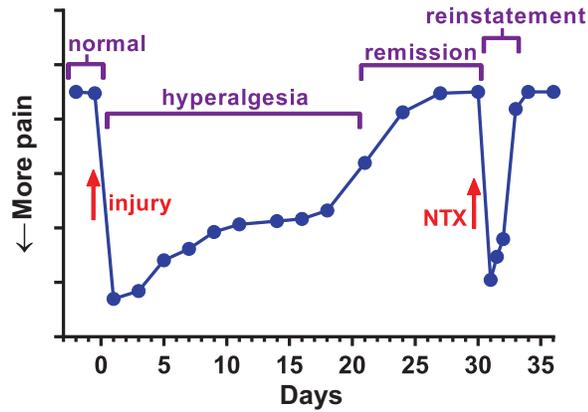
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## INTRODUCTION

Many types of chronic pain, and in particular neuropathic pain, are episodic: periods of pain are interspersed with periods of remission, which resemble the healthy state but can quickly give way to yet another bout of pain. In particular, stress is a common trigger of pain episodes in these disorders. Recently, a rodent model of chronic pain has been developed that reproduces both its episodic nature and its sensitivity to stress. It has been called latent pain sensitization (Bessiere et al., 2007; Campillo et al., 2011) or latent sensitization (Lian et al., 2010); here we will use the later term abbreviated to LS. Figure 9.50.1 illustrates the following basic phases of LS:

1. Tissue injury. This leads from the normal state to a period of hyperalgesia (increased responses to noxious stimuli) or allodynia (pain-like responses to a nonnoxious stimulus). A variety of noxious stimuli can induce LS in rodent models of chronic pain, including a paw incision (Li et al., 2001; Richebe et al., 2005; Rivat et al., 2007; Campillo et al.,





**Figure 9.50.1** The phases of latent sensitization (LS). (A) Tissue injury leads from the normal state into a period of hyperalgesia. (B) Pain subsides into a remission phase. (C) An injection of opioid inverse agonist (naltrexone, NTX) produces a temporary reinstatement of the hyperalgesia. (2011), complete Freund's adjuvant (CFA; Corder et al., 2013), carrageenan (Bessiere et al., 2007; Le Roy et al., 2011), and nerve injury (Solway et al., 2011).

2. Remission of hyperalgesia. The initial hyperalgesia eventually subsides into a period of remission. The length of the initial hyperalgesic phase depends on the injury, ranging from 7 days after paw incision (Li et al., 2001) to ~100 days in the cuff model of neuropathic pain (Yalcin et al., 2011).

3. Reinstatement of hyperalgesia. However, the remission phase does not represent a return to the normal state, as the hyperalgesia can be reinstated by a variety of stimuli or pharmacological agents. The most commonly used are antagonists of the  $\mu$ -opioid receptor (MOR) such as naloxone or naltrexone (NTX), which reinstate the hyperalgesia for a period of time that is consistent with the pharmacokinetic half-life of the drug (2 to 4 hr). Strictly speaking, naloxone and naltrexone are inverse agonists of the MOR (see Background Information). NTX does not produce hyperalgesia in naïve animals. Of note, reinstatement by NTX can be repeated any number of times over a period of at least 5 months (Campillo et al., 2011; Corder et al., 2013). Other stimuli such as stress can similarly reinstate the painful state (Le Roy et al., 2011). The Basic Protocol describes how to induce LS in mice by injecting CFA in the hind paw. The Alternate Protocol provides a similar procedure to use in rats. The Support Protocol describes the use of von Frey filaments, one of the methods most commonly used to measure hyperalgesia in the different phases of LS.

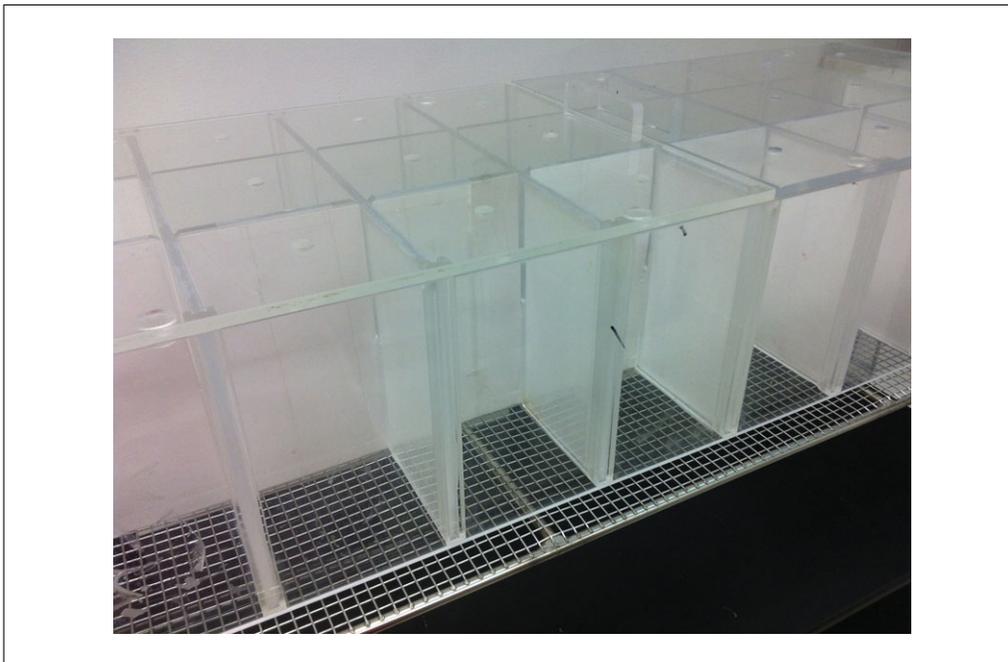
## BASIC PROTOCOL

### COMPLETE FREUND'S ADJUVANT-INDUCED LATENT SENSITIZATION IN MICE

LS induced by injecting CFA in the hind paw of mice is a robust, well characterized instance of this pain model, so we have chosen it as an example. This protocol can be adapted to study LS induced with other stimuli by simply changing the stimulus and paying attention to the different duration of the hyperalgesic phase. For mice, some groups have found that handling increases rather than decreases struggling, and therefore, they avoid habituation. We have included here instructions for habituation (as performed in our laboratory) in case the investigator decides to use it.

#### Materials

6-week-old to 5-month-old female or male mice, 20 g and 24 g, respectively (e.g., C57Bl/6 J, Jax Mice, The Jackson Laboratory), five to eight mice per group per gender



**Figure 9.50.2** Acrylic enclosure for measuring paw withdrawal responses to von Frey filaments in mice.

PicoLab Rodent Diet 20 (LabDiet, cat. no. 5053) and water bottles  
 Complete Freund's adjuvant (CFA; Sigma, cat. no. F5881)  
 Naltrexone hydrochloride (Sigma, cat. no. N3136) *or* naloxone hydrochloride dihydrate (Sigma, cat. no. N7758)  
 Phosphate-buffered saline (PBS; e.g., see *APPENDIX 2A*)  
 Water, sterile  
 Isoflurane (Phoenix)

~30 cm × 15-cm polycarbonate mouse cages for up to five mice per cage  
 Animal room with control of light (30 to 50 Lux), ambient temperature (18° to 26°C), and relative humidity (30% to 70%)  
 von Frey filaments (see Support Protocol)  
 10.16 × 10.16-cm opaque acrylic enclosures on top of an elevated mesh metal grid with stand (IITC, CA, Part number 410), large enough to test multiple mice (Fig. 9.50.2)  
 50- $\mu$ l Hamilton syringe (Hamilton, cat. no. 7637-01) with 30-G needle.  
 Vaporizer for isoflurane, with induction box (Summit Anesthesia Support)

Additional reagents and equipment for measuring PWTs with von Frey filaments (Support Protocol) and for anesthetizing (*APPENDIX 4B*; Davis, 2008) and euthanizing (*APPENDIX 4H*; Donovan and Brown, 2005) mice

#### ***Habituate mice (optional)***

1. Handle each 6-week-old to 5-month-old female or male Jax mouse 5 min/day for ~1 week before starting the experiment. Avoid holding the mice by the tail, and train the mice to walk onto an outstretched hand.

*We have found other strains to be adequate. From experience, we have found that a sample size of five to eight mice per group per gender is sufficient to reduce error and obtain statistical significance. Mice are typically tested as young as 6 weeks and as old as 5 months. They are usually littermates or age-matched.*

2. Three days before the first test, habituate mice in their home cages to the testing room with dim lighting (30 to 50 Lux).

*The habituation procedures vary among labs, depending on the rules of the facility. The mice may be moved back to the housing room or housed in the testing room with IACUC approval.*

3. Habituate the mice to the testing apparatus (enclosure) 30 min daily for 2 days.

#### **Take baseline measurements**

4. Allow the mice to acclimate in the acrylic enclosures atop the elevated grid for at least 30 min before testing. If the enclosure is less than 10 to 12 in. high, put a cover on the enclosures so that the mice cannot jump out.

*To reduce social visual cues from one mouse to another, the enclosures should not be translucent. Laminated cards (white or black) can be fitted to the enclosures. See photo (Fig. 9.50.2). Tall acrylic cylindrical tubes, 10 cm in diameter, can be used instead of square boxes.*

5. Measure baseline paw withdrawal thresholds (PWTs) with von Frey filaments once a day for 2 to 3 days before CFA injection by gently applying the von Frey filaments, between the openings of the grid, to the soft pad of the hind paw between the tori at the base of the digits. Use the up-and-down method (see Support Protocol).
6. Avoid taking measurements while the animal is standing on its hind legs, grooming, or sleeping. If sleeping, very gently nudge the trunk with a pen. When testing multiple animals, move from animal to animal depending on the activity level.
7. Return animals to their cages and usual housing room after the measurements.
8. Clean the rack and the area below it with deionized water between experiments.

#### **Inject CFA for the hyperalgesia phase**

9. Take the final baseline measurements immediately before the CFA injection, and record them as day 0.

*We do not average baseline values but analyze them statistically to detect changes over time (3 to 4 days) and between experimental groups. Baseline measurements need to be analyzed carefully because we have detected differences in baseline responses in two strains of KO mice.*

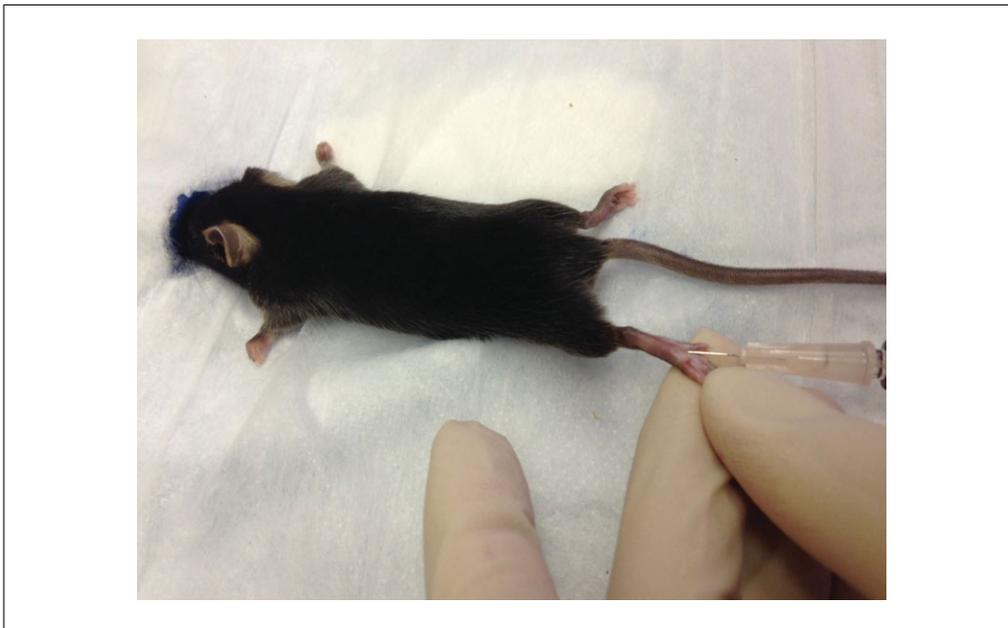
10. Anesthetize the animals (APPENDIX 4B; Davis, 2008) in a supine position with 1% to 2% isoflurane using the vaporizer and induction box.
11. Mix the CFA thoroughly before each injection (it tends to settle at the bottom of the bottle), and draw it directly into a 50- $\mu$ l Hamilton syringe with a 30-G needle.

*CFA can be used undiluted (100%) and injected subcutaneously in one hind paw. Others inject it as a 1:1 emulsion in water, but this requires twice the injection volume.*

*The amount of CFA is a critical parameter; we suggest 50  $\mu$ l undiluted for rats and 5  $\mu$ l undiluted for mice to yield a robust hyperalgesia that resolves fairly quickly.*

12. Insert the needle at an oblique angle of  $\sim 20^\circ$ , at the middle of the dorsal paw, near the base of the third toe (Fig. 9.50.3). Slowly inject CFA over 1 to 2 sec. Hold the needle in place for 5 to 15 sec to allow pressure to dissipate, and then withdraw it gently.
13. Measure PWTs the next day and on selected subsequent days (see Support Protocol).

*Do not perform PWTs daily, as this may lead to stress-induced hyperalgesia. We typically measure PWTs on days 1, 3, 5, 7, 10, 14, 21, 28 after the CFA injection. This timeline*



**Figure 9.50.3** Injection of CFA in the hind paw of a mouse.

*includes the initial phase of peak hyperalgesia (days 1 to 3), gradual resolution of initial hyperalgesia (days 5 to 21), and then the remission phase (day 21 onwards, Fig. 9.50.1).*

*PWTs should decrease dramatically on days 1 and 3, and then progressively return to baseline values, which should occur between days 14 and 28. Note that inflammation of the paw will develop quickly within the first few hours and days after CFA injection and then persist for several weeks. During this time, animals may exhibit extended periods of paw elevation, thus precluding testing.*

#### ***Inject naltrexone for the pain reinstatement phase***

14. Measure PWTs before naltrexone is injected.

*This will serve a baseline to compare responses after naltrexone injection.*

15. Dissolve naltrexone in sterile phosphate-buffered saline (PBS) the same day it is used, and inject 3 mg/kg in 300  $\mu$ l subcutaneously at the nape.
16. Measure PWTs intermittently, e.g., at 20, 40, 60, and 120 min, or 5, 15, 30, 45, 60, 90, 120, 180, and 240 min, and then again at 24 hr to ensure that there is a return to baseline.

*An alternative protocol with fewer PWT measurements and more time in the home cage can be followed to reduce test- or stress-induced hyperalgesia. Measure a baseline PWT 24 hr prior to the NTX injection. Habituate the mice to the testing room in their home cages for 15 min. Inject NTX and replace the mice in the home cage for 10 min. Then place them into the enclosure for an additional 10 min, and measure PWTs at 20 and 60 min after injection.*

#### ***Sacrifice animals and measure endpoints***

17. At the end of the experiment, euthanize the mice with an overdose of isoflurane (5%) and cervical dislocation (APPENDIX 4H; Donovan and Brown, 2005).

*An overdose of pentobarbital (100 mg/kg) can also be used to euthanize the mice.*

*Tissues can be taken for physiological, biochemical, or molecular analyses.*

*Endpoints can vary, depending on the particular study.*

## **COMPLETE FREUND'S ADJUVANT-INDUCED LATENT SENSITIZATION IN RATS**

The protocol in rats is basically the same as in mice. Note the different range of force of the von Frey filaments and the different needle used to inject naltrexone. If the same equipment is used for mice and rats, it is critically important to wash it thoroughly to eliminate all odor of the previous species, which can be a stressor. Habituation to handling and to the testing equipment reduces variability of the data collected in rats.

### ***Materials List***

250- to 300-g male Sprague-Dawley rats (Harlan), five to eight rats per group per gender, typically three per cage

Rat chow (e.g., LabDiet) and water bottles

Complete Freund's adjuvant (CFA; Sigma, cat. no. F5881)

Naltrexone hydrochloride (Sigma, cat. no.) N3136 *or* naloxone hydrochloride dihydrate (Sigma, cat. no, N7758)

0.9% (w/v) saline, sterile

Water, sterile

Isoflurane (Phoenix)

Pentobarbital (e.g., Fatal-Plus, Vortech Pharmaceuticals)

~20 × 33-cm polycarbonate rat cages

Animal room with control of light (30 to 50 Lux), ambient temperature, and relative humidity

10.16 × 20.3-cm opaque acrylic enclosures on top of an elevated mesh metal grid with stand (IITC, part no. 410) large enough to test multiple mice

50- $\mu$ l Hamilton syringe, 26-G needle

Vaporizer for isoflurane, with induction box (Patterson Scientific)

Additional reagents and equipment for measuring baseline paw withdrawal thresholds (PWTs) with von Frey filaments (Support Protocol) and anesthetizing (*APPENDIX 4B*; Davis, 2008) and euthanizing rats (*APPENDIX 4H*; Donovan and Brown, 2005)

### ***Habituate rats (recommended)***

1. Habituate rats to the testing apparatus for 2 days, 30 min daily (see Basic Protocol).

*Rats are housed three per cage unless they have intrathecal catheters, in which case they are housed individually.*

### ***Take baseline measurements***

2. Allow the rats to acclimate in the acrylic enclosures atop the elevated grid for at least 30 min before testing.
3. Measure baseline PWTs with von Frey filaments for 2 to 3 days before the CFA injection by gently applying the von Frey filaments, between the openings of the grid, to the soft pad of the hind paw between the tori at the base of the digits. Use the up-and-down method (see Support Protocol).
4. Avoid taking measurements while the animal is standing on its hind legs, grooming or sleeping. If sleeping, very gently nudge the trunk with a pen.
5. Return animals to their cages and usual housing room after the measurements.
6. Clean the rack and the area below it with deionized water between experiments.

### ***Inject CFA for the hyperalgesia phase***

7. Take the final baseline measurements just before the CFA injection, and record as day 0.
8. Anesthetize the rats (*APPENDIX 4B*; Davis, 2008) in the induction box with 5% isoflurane, supplied by the vaporizer.
9. Mix undiluted CFA thoroughly before each injection, and draw 50  $\mu$ l directly into the 1-ml Hamilton syringe with a 25-G needle.

*CFA is used undiluted (100%), and the injection volume is a critical parameter. Use 50  $\mu$ l for rats.*

10. Insert the needle at an oblique angle from the heel in the middle of the paw, and inject the CFA. Hold the needle in place for 15 sec to allow pressure to dissipate, and then withdrawn gently.
11. On the following day, measure PWTs and on subsequent days as appropriate. Do not perform PWTs daily as this may lead to stress-induced hyperalgesia.

*We typically measure PWTs on days 1, 3, 5, 7, 14, 21, 28 after the CFA injection. This would assess the initial (days 1 to 7) and later (day 7 to 21) response, followed by the full recovery phase (day 21 onwards).*

*The PWTs should decrease dramatically on day 1 and progressively return to baseline values, which should occur between days 20 and 30.*

### ***Inject naltrexone for the pain reinstatement phase***

12. Measure PWTs just before the naltrexone injection.

*This will serve a baseline to compare responses after naltrexone injection.*

13. Dissolve the naltrexone the same day in sterile saline, and subcutaneously inject 1 mg/kg in 300  $\mu$ l or intrathecally inject 1  $\mu$ g (2.6 nmol) in 10  $\mu$ l, plus an intrathecal 10  $\mu$ l flush.
14. Measure PWTs intermittently, e.g., at 15, 30, 45, 60, 90, and 120 min.

### ***Sacrifice animals and measure endpoints***

15. At the end of the experiment, euthanize the rats with an overdose (100 mg/kg) of pentobarbital (*APPENDIX 4H*; Donovan and Brown, 2005).

## **UP-AND-DOWN METHOD OF von FREY MEASUREMENTS**

The up-and-down method using von Frey hairs has become the most common approach to the measurement of mechanical paw hypersensitivity after tissue or nerve injury. However, several other procedures are available for applying von Frey hairs, and several other stimulus paradigms can be used, e.g., the use of an electronic von Frey apparatus (Parada et al., 2003).

### ***Materials***

Treated mice (Basic Protocol) or rats (Alternate Protocol)

Set of eight von Frey filaments

For mice (Touch-test; North Coast Medical):  $\text{Log}_{10}$  [10\*force (mg)] or (g) = 1.65 (0.008 g), 2.36 (0.02 g), 2.83 (0.07 g), 3.22 (0.61 g), 3.61 (0.4 g), 4.08 (1.0 g), 4.31 (2.0 g), 4.74 (6.0 g) *or*

For rats (Touch-test; North Coast Medical):  $\text{Log}_{10}$  [10\*force (mg)] = 3.61 (0.4 g), 3.84 (0.6 g), 4.08 (1.0 g), 4.31 (2.0 g), 4.56 (4.0 g), 4.74 (6.0 g), 4.93 (8.0 g), 5.18 (15.0 g)

## **SUPPORT PROTOCOL**

**Preclinical Models  
of Neurologic and  
Psychiatric  
Disorders**

### **9.50.7**

Date:										
Exp:										
Mouse #	Fiber #	1	2	3	4	5	6	7	8	9
1	1 (1.65)									
	2 (2.36)									
	3 (2.83)									
	4 (3.22)	O		O						
	5 (3.61)		X		O					
	6 (4.08)					O				
	7 (4.31)						X			
	8 (4.74)	50% threshold: 0.573								
2	1 (1.65)									
	2 (2.36)									
	3 (2.83)									
	4 (3.22)	O								
	5 (3.61)		O							
	6 (4.08)			O						
	7 (4.31)				O		O		O	
	8 (4.74)					X		X		X
3	1 (1.65)									
	2 (2.36)									
	3 (2.83)									
	4 (3.22)	O								
	5 (3.61)		O							
	6 (4.08)			O		O				
	7 (4.31)				X		O		O	
	8 (4.74)							X		
4	1 (1.65)				O		O			
	2 (2.36)			X		X				
	3 (2.83)		X							
	4 (3.22)	X								
	5 (3.61)									
	6 (4.08)									
	7 (4.31)									
	8 (4.74)	50% threshold: 0.007								
5	1 (1.65)									
	2 (2.36)									
	3 (2.83)									
	4 (3.22)	X		O						
	5 (3.61)				O			O		
	6 (4.08)						X			
	7 (4.31)									
	8 (4.74)	50% threshold: 0.426								
6	1 (1.65)									
	2 (2.36)				O		O			
	3 (2.83)			X		X		X		
	4 (3.22)	X								
	5 (3.61)									
	6 (4.08)									
	7 (4.31)									
	8 (4.74)	50% threshold: 0.032								
7	1 (1.65)					O		X		
	2 (2.36)				X		X			
	3 (2.83)			X						
	4 (3.22)	X								
	5 (3.61)									
	6 (4.08)									
	7 (4.31)									
	8 (4.74)	50% threshold: 0.003								
8	1 (1.65)									
	2 (2.36)									
	3 (2.83)		O		O			O		
	4 (3.22)	X		X		X				
	5 (3.61)									
	6 (4.08)									
	7 (4.31)									
	8 (4.74)	50% threshold: 0.112								

**Figure 9.50.4** Sample scoring sheet for the up-and-down method of von Frey measurements. Examples of responses of eight different mice. X represents a paw withdrawal response, and O represents failure to respond. Numbers in parentheses are  $\log_{10} [10 \times \text{force (mg)}]$ . Final 50% threshold values were calculated using the algorithm used by Chaplan et al. (1994).

In-house up-and-down scoring sheet (e.g., see Fig. 9.50.4)

- 1a. *For mice:* For the first trial, gently apply the 3.22 (0.61 g) filament between the openings of the grid, to the soft pad of the hindpaw between the tori at the base of the digits for 3 sec.
- 1b. *For rats:* For the first trial gently apply the 4.31 (2.0 g) filament between the openings of the grid, to the soft pad of the hindpaw between the tori at the base of the digits for 4 sec.

*The exact location depends on the pain model. In animal models of paw inflammation such as CFA, place the filament on the centermost region of the footpad, and apply sufficient pressure to cause a slight bend in the filament. Apply pressure gently, as a rapid increases in force can quickly sensitize peripheral nerves, yielding a false positive response.*

*Ensure that the amount of pressure applied to the paw remains constant for 4 sec in the rat or 3 sec in the mouse.*

2. If the paw withdraws briskly, record as a positive response (mark an X on the appropriate column on the up-and-down scoring sheet), and then apply the next lowest von Frey filament. If the paw does not respond, record as a negative response (mark an O in the appropriate column), and then apply the next highest von Frey filament.

- Record four additional measurements after the first change in response (negative to positive or positive to negative).

*Examples are mouse 1, 5, and 8 on the sample scoring sheet (Fig. 9.50.4).*

*A positive response is defined as an abrupt lifting of the paw that is not due to normal walking or grooming.*

- Do not record a value if:
  - the filament slips off of the paw before a withdrawal
  - the filament engages the abdomen, some other sensitive area, or the wrong area of the paw
  - the filament touches the wire mesh
  - the animal moves (walks or grooms).

In all these cases, give the animal a 30-sec break before retesting with the same fiber.

- For mice not responding to any filament:* Assign the reaction to the 4.74 fiber, the fiber of the highest gram force, as a positive response.

*See mice numbers 2 and 3 in the sample scoring sheet (Fig. 9.50.4).*

- For rats not responding to any filament:* Assign a maximum value of 15 g.

- For mice responding to all filaments:* Assign animals a minimum value of 0.005 g.

*For example, see mouse number 4 on the sample scoring sheet (Fig. 9.50.4).*

- For rats responding to all filaments:* Assign animals a minimum value of 0.5 g.

*In the case of mice, another complex scenario is a positive response on the first 2 fibers. In this case, we have adapted the protocol for a total of 6 measures. This is to avoid repeated testing of a mouse that appears sensitized. See mice numbers 4, 6, and 7 in the sample scoring sheet (Fig. 9.50.4).*

- When all measurements have been taken, input the X and O values into the algorithm for the up-and-down method (Chaplan et al., 1994). Make sure to include all preceding O values to the first response on the score sheet and in the algorithm.

## COMMENTARY

### Background Information

#### *A brief history of LS*

LS was initially found to develop in the setting of opiate-induced hyperalgesia, triggered by the repeated administration of opiates like heroin, morphine (Celerier et al., 2000; Li et al., 2001), fentanyl (Bessiere et al., 2007; Rivat et al., 2007; Rivat et al., 2009), and remifentanyl (Campillo et al., 2011). Opiate-induced and tissue injury-induced hyperalgesia are additive (Campillo et al., 2011) and share several characteristics: they are reinstated by naloxone (Campillo et al., 2011), seem to involve the activation of neurokinin 1 (NK1) receptors and descending pain control pathways (Rivat et al., 2009), and can be blocked by opioid or NMDA receptor antagonists (Rivat et al., 2007; Campillo et al., 2011; Le Roy et al., 2011).

In neuropathic pain and other chronic pain disorders, pain episodes are often triggered by stress. LS models replicate this characteristic; forced swim stress and novel environment stress can produce reinstatement when given during the remission phase (Rivat et al., 2007), just like opioid inverse agonists. Hyperalgesic priming (Reichling and Levine, 2009; Joseph et al., 2010) is a model similar to LS in that it is long lasting (>3 weeks), the original hyperalgesia can be reinstated (in this case by pronociceptive agents such as prostaglandin E<sub>2</sub>) and is increased by stress. Whereas hyperalgesic priming takes place in peripheral afferent terminals, LS seems to be mediated centrally (Solway et al., 2011; Corder et al., 2013), although LS caused by nerve injury may be also mediated peripherally (Guan et al., 2010). Therefore, the relationship between hyperalgesic priming and LS remains unclear.

Mechanistically, both the hyperalgesia and reinstatement phases involve activation of *N*-methyl-D-aspartate receptors (NMDARs; Rivat et al., 2007; Campillo et al., 2011; Corder et al., 2013), whereas the remission phase involves activation of MORs (Corder et al., 2013) or  $\kappa$ -opioid receptors (KORs; Campillo et al., 2011). Although the location of opioid receptors involved in LS include the spinal cord, it is not known whether these are in dorsal horn neurons, central terminals of primary afferent, or both. Other questions to resolve are the mechanism underlying induction and maintenance of the prolonged activation of opioid receptors during the remission phase. The most obvious mechanism is the tonic release of opioid peptides in the dorsal horn. However, recent evidence suggests that remission involves constitutive signaling of MORs in the dorsal horn that silences LS and thus maintains an analgesic state (Corder et al., 2013).

#### *$\mu$ -opioid receptors*

MORs are members of the Class A of G protein-coupled receptors (GPCRs) that are  $G_{i/o}$  coupled and, for the most part, inhibit their cognate second messenger signaling pathways. This results in an inhibition of adenylyl cyclase and ion channels, but activation of components of the MAP kinase cascade. As MORs bind morphine, the most clinically effective analgesic, ligand-induced signaling of MORs has been extensively studied. We know that when a MOR binds its agonist, it is phosphorylated by kinases such as GRK2/3, PKA, PKC, CaMKII, and Src, which then recruit  $\beta$ -arrestin 1 or 2. The receptor is then internalized, rather than degraded, through a clathrin-dependent pathway and recycled through a Rab11 pathway.

In addition to such ligand-dependent signaling, MORs may signal in the absence of agonist (constitutive activity). This ligand-independent signaling state was first described for the  $\delta$ -opioid receptor by Costa and Herz (1989) and relied on a pharmacological approach to detect negative intrinsic activity of GPCRs. Using an array of inverse agonists and neutral antagonists, constitutively active MORs have also been found (Wang et al., 1994). Unlike  $\delta$ -opioid receptors, these receptors do not comprise a major proportion of the total receptor population under basal conditions (Vezei et al., 2013). However, withdrawal from chronic morphine increases constitutively active MORs in rodents (Wang et al., 1994; Shoblock and Maidment,

2006; Shoblock and Maidment, 2007; Wang et al., 2007; Meye et al., 2012) and enhances the aversive effect of naloxone, a MOR inverse agonist, in the morphine dependent state (Shoblock and Maidment, 2006).

LS also increases constitutively active MORs to enhance endogenous analgesia and physical dependence (Corder et al., 2013). Our understanding of this signaling state is limited, but it was recently reported that constitutive activation of PKC $\alpha$  results in MOR phosphorylation at Ser<sup>363</sup> (Illing et al., 2014) and that constitutively active receptors are rapidly internalized through a c-Src- and  $\beta$ -arrestin-2-dependent mechanism (Walwyn et al., 2007; Lam et al., 2011). As the LS model involves a substantial increase in constitutive activity of MORs, it provides a unique opportunity to study this interesting signaling state. A range of questions about the pathways and molecules that activate and maintain constitutive activity and the affected receptor populations, cell types, and downstream signaling pathways remain to be determined. We posit that there must be intrinsic mechanisms to reverse LS; otherwise, any individual exposed to a severe injury would be in a state of LS. Determination of such intrinsic mechanisms could yield clues leading to cures for chronic pain.

#### *Inverse agonists and neutral antagonists*

Constitutive activity of a receptor consists of an agonist-independent increase in signaling. Compounds that decrease this signal are called inverse agonists. There are also compounds that bind to the receptor without affecting their constitutive activity, yet they eliminate both receptor activation by agonists and the inhibitory effect of the inverse agonists; these compounds are called neutral antagonists. To establish the presence of constitutive activity, it is necessary to assess the following: (1) that an inverse agonist decreases the basal signaling of the receptor and (2) that this effect of the inverse agonist disappears in the presence of a neutral antagonist. Constitutive activity of receptors other than MORs may contribute to pain remission (e.g., cannabinoid receptors), and the study of these receptors requires the availability of appropriate inverse agonists and neutral antagonists.

#### **Critical Parameters**

Injury-induced LS is robust and easily reproducible in the laboratory. This basic protocol of CFA-induced LS can be easily adapted

to study other types of chronic pain by changing the insult that triggers the hyperalgesia.

### ***Injuries used to elicit latent sensitization***

Most tissue injuries that induce persistent pain (i.e., pain that lasts several days or more) have been found to induce LS. Stimuli that have been reported to induce LS in rodents include plantar incision (Li et al., 2001; Richebe et al., 2005; Rivat et al., 2007; Campillo et al., 2011; Romero et al., 2011; Corder et al., 2013), CFA (Solway et al., 2011; Corder et al., 2013), carrageenan (Bessiere et al., 2007; Le Roy et al., 2011), visceral pain (Lian et al., 2010), and nerve injury (Solway et al., 2011). In addition, opiate drugs like morphine, fentanyl, and remifentanyl can induce LS by themselves and have synergistic effects with the injury stimuli listed above (Celerier et al., 2000; Li et al., 2001; Laulin et al., 2002; Richebe et al., 2005; Rivat et al., 2009; Campillo et al., 2011).

The choice of the stimulus used in a particular LS study would depend largely on the questions and hypotheses being considered. The first step in such a study would be to confirm that the chosen injury produces a consistent, measurable indicator of hyperalgesia or allodynia. Not all injuries produce persistent pain, and not all forms of pain elicit measurable behavioral responses. Conversely, of particular concern are models that produce a very long-lasting period of hyperalgesia. For example, some models of peripheral neuropathic pain produce hyperalgesia lasting 80 days, as in the cuff nerve injury model (Yalcin et al., 2011), or even longer, as in the spared nerve injury model (Decosterd and Woolf, 2000). Therefore, to study LS related to neuropathic pain, modifications must be made to reduce the severity of the nerve injury. We recommend a modified version of the spared nerve injury model, the  $Cp_xS_x$  model (Shields et al., 2003; Solway et al., 2011), in which the common peroneal and sural branches of the sciatic nerve are cut and the tibial branch is left intact.  $Cp_xS_x$  produced a hyperalgesic phase lasting ~28 days in mice (Solway et al., 2011) and ~35 days in rats (J.C. Marvizon, unpublished observations).

### ***Methods for measuring hyperalgesia and allodynia***

Whereas the method using von Frey filaments described here measures mechanical hypersensitivity, other methods can be substituted to measure heat or cold hypersensitivity. Thus, Solway et al. (2011) measured not only tactile hypersensitivity using von Frey

filaments but also cold hypersensitivity upon topical application of a drop of acetone to the plantar paw skin. Other studies (Li et al., 2001) have measured heat hyperalgesia using paw withdrawal responses to radiant heat (Hargreaves et al., 1988). One study on LS to visceral pain (Lian et al., 2010) measured referred visceral hypersensitivity in rats by applying von Frey filaments to the lumbar dermatomes.

### ***Drug administration to trigger reinstatement***

In addition to inverse agonists of MORs, antagonists of other receptors can also trigger reinstatement. For example, nornalorphimine, a  $\kappa$ -opioid receptor antagonist, produces reinstatement in LS induced by plantar incision (Campillo et al., 2011). Similarly, antagonists of Y1 or Y2 receptors for neuropeptide Y produced reinstatement to LS induced by nerve injury ( $Cp_xS_x$ ) or intraplantar CFA injection (Solway et al., 2011).

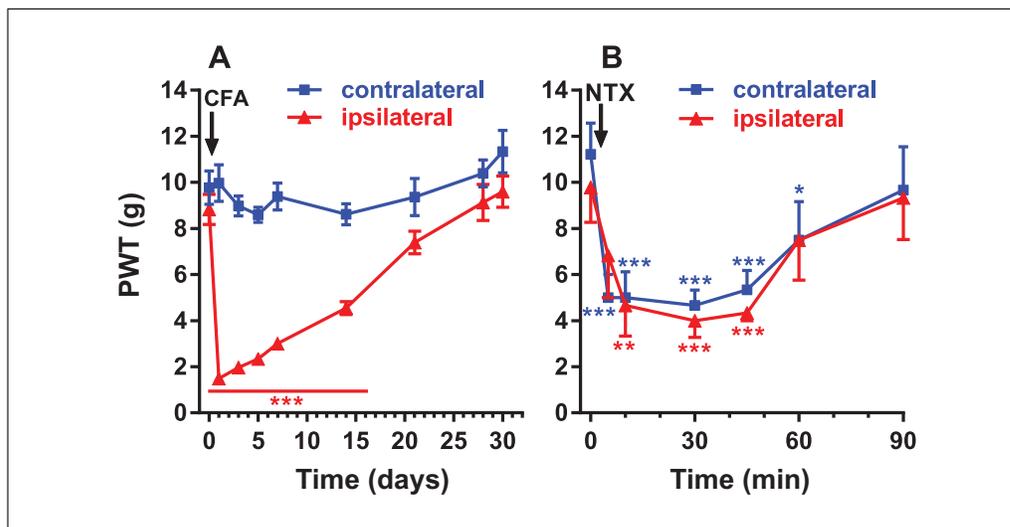
The route of drug administration is another important variable. Systemic injections of compounds that cross the blood-brain barrier can potentially affect receptors anywhere in the body. As LS seems to be partially mediated by supraspinal mechanisms (Rivat et al., 2009; De Felice et al., 2011; Le Roy et al., 2011; Taylor and Corder, 2014), this leads to uncertainty about the site of action of the drug and the location of the receptors involved following systemic administration. To determine whether the receptors involved are located in the spinal cord, drugs can be injected intrathecally. However, the use of surgically implanted, chronic intrathecal catheters could cause problems with interpretation of results, because the surgical procedure used to implant the catheter may cause an injury response leading to the development of LS. Hence, LS may be present in control animals that received a nonnoxious stimulus (for example, saline instead of CFA) due to catheter implantation surgery. To avoid this problem, we recommend that intrathecal injections be conducted using an acute percutaneous method (Hylden and Wilcox, 1980). Alternatively, a control group of animals without intrathecal catheters could be included in the experiment.

### **Troubleshooting**

Table 9.50.1 includes a list of potential problems and possible solutions. It is very important to establish a reliable baseline of responses before applying the injury stimulus that induces LS; otherwise, it will be difficult to determine whether the thresholds of the animals have returned to baseline. PWT values

**Table 9.50.1** Troubleshooting Guide for the Latent Sensitization Model of Chronic Pain

Problem	Possible causes and solutions
Injurious stimulus does not produce hyperalgesia	<p>If using CFA, check that it is fully dissolved.</p> <p>Check the procedures involved in producing the stimulus (e.g., surgery to induce nerve injury).</p> <p>If using a novel stimulus, change the variables related to the stimulus until hyperalgesia is reliably induced.</p> <p>The stimulus used may not be adequate to induce chronic pain; search the literature for a stimulus that induces the desired type of hyperalgesia.</p>
Hyperalgesic phase too short (<3 days)	The stimulus used may not be adequate to induce chronic pain; try increasing the severity of the injury.
Hyperalgesic phase too long (>1 month)	<p>A common problem with nerve injury models; consider switching to the Cp<sub>x</sub>S<sub>x</sub> nerve injury model.</p> <p>Decrease the extent of the nerve injury.</p> <p>When using inflammatory compounds like CFA, decrease the volume of injection.</p>
Responses plateau, but below initial baseline	<p>Baseline not adequately established; try repeating measurements of baseline on different days to obtain low variability.</p> <p>Result may be valid and not artifactual, possibly because this particular group of animals cannot activate MORs or other receptors to compensate for hyperalgesia; report these findings.</p>
Baselines different between groups of animals	<p>Randomly assign animals to each group.</p> <p>Be sure to alternate between groups when establishing baselines and to measure behavior under the same conditions.</p>
Hyperalgesia detected both ipsilateral and contralateral to the stimulus	<p>LS may have been induced by another stimulus, e.g., intrathecal catheter implantation or other surgery; include controls to determine if such stimuli are able to induce latent sensitization.</p> <p>Animal may have pre-existing LS due to an undetected injury; discard this particular animal.</p> <p>If found consistently in the same group of animals, result is probably valid; may be caused by spreading of the sensitization to the contralateral dorsal horn or to supraspinal mechanism; reinstatement of hyperalgesia often occurs bilaterally.</p>
Paw responses (to von Frey hairs or other measures) become higher than baseline	<p>Sometimes observed in the contralateral side after nerve injury; results are probably valid, possibly because of a strong anti hyperalgesic mechanism that overshoots the basal state; report these findings.</p> <p>Baseline may not have been adequately established; more baseline measurements that are stable over time may be needed.</p>
Naltrexone or naloxone do not produce reinstatement	<p>Prepare fresh solutions daily.</p> <p>Check method of injection, particularly if intrathecal; percutaneous intrathecal injections require training.</p>
No return to baseline after injecting drug to produce reinstatement	<p>Some <math>\kappa</math>-opioid receptor antagonists have extremely slow dissociation rates, to the point of being pseudoirreversible; keep measuring responses daily to find out when they return to baseline.</p> <p>Injection procedure has produced an injury or sensitization; the experiment may need to be repeated with a more careful injection of drug.</p>
Reinstatement occurs spontaneously or after saline injection	Likely, the animal has been stressed, perhaps by the animal handler during injection (quite common with new investigators), which produces strong reinstatement; provide additional training to achieve rapid, minimally stressful handling methods.
During the remission phase, responses drift up or down	<p>Animals may become habituated or sensitized if pain measures are done too frequently; allow more time (e.g., 1 week) between the pain measurements.</p> <p>Drugs injected to produce reinstatement may induce subtle long-term effects; careful investigation using adequate controls is merited.</p>



**Figure 9.50.5** Experiment showing CFA-induced LS in rats. (A). Rats ( $n = 13$ ) received an injection of CFA ( $50 \mu\text{l}$ , subcutaneously) in one hindpaw. Mechanical hypersensitivity, measured with von Frey hairs, developed ipsilaterally and resolved by day 28. Two-way ANOVA:  $p < 0.0001$  for time, side and interaction,  $p = 0.0002$  for subject matching. (B). On day 30, six of the rats were subcutaneously injected with  $1 \text{ mg/kg}$  naltrexone (NTX), which resulted in reinstatement of hypersensitivity lasting  $\sim 1 \text{ hr}$ . Two-way ANOVA:  $p < 0.0001$  for time and subject matching,  $p = 0.96$  for side,  $p = 0.65$  for interaction. Holm-Sidak's post-hoc tests:  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

greater than baseline (indicating the presence of analgesia) have been observed, but this effects tends to be small and thus requires a carefully established and reliable baseline. For these reasons, we recommend that baseline responses be assessed over multiple days, until stable. Another confounding factor is stress, which can produce a reinstatement that is as robust as that produced by naltrexone. Indeed, the presence of humans (men in particular) can cause stress in rodents and change their behavioral responses to a noxious stimulus (Sorge et al., 2014). Therefore, testing should be completed by the same person in each experiment.

Some laboratories do not repeatedly habituate mice to the testing equipment, although this is almost always recommended in rats. Unlike rats, mice do not necessarily habituate and may become even more stressed by the handling.

### Anticipated Results

Figure 9.50.5 shows the results of a representative experiment in which LS was induced by injecting CFA ( $50 \mu\text{l}$  subcutaneous) in the hind paw of rats, as described above. Notice the robust hypersensitivity in the ipsilateral side, which resolved after 28 days (Fig. 9.50.5A). Responses to von Frey filaments in the contralateral side remained at baseline. On day 30, rats were injected subcutaneously with  $1 \text{ mg/kg}$  naltrexone. This

resulted in reinstatement of hyperalgesia for  $\sim 1 \text{ hr}$  in the ipsilateral side and the emergence of hyperalgesia in the contralateral side (Fig. 9.50.5B).

### Time Considerations

When used as a model of chronic pain, LS experiments last weeks and thus require maintenance of animals in a controlled environment for extended periods of time. This leads to higher animal costs and more complex institutional animal care protocols, which must be considered. However, once LS has been induced, animals require only standard care and occasional behavior measurements. With adequate planning to stagger measures, a single investigator can handle a large number of animals. However, the interval between each measurement and the time taken to test a batch of mice must be taken into consideration. The number of mice being tested in one batch cannot exceed the time interval for acquiring one set of measurements. In this case, good record keeping and animal identification methods are essential.

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### Conflict of Interest

The authors have no conflict of interests.

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