Assessment of Acute Thermal Nociception in Laboratory Animals

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Summary

Models of acute nociception using a thermal stimulus are widely employed as screening methods for nociceptive properties of new drug compounds. In this chapter, detailed descriptions for conducting of two of the most commonly used models; the hot plate test and the “Hargreaves test,” are described. These models are applicable to both rats and mice and have the advantage of allowing repeated and multiple testing using a single animal because the stimulus is transitory and produces no tissue damage. Additionally, a modification of these models using a skin-twitch reflex that is applicable to large laboratory animals such as dogs or sheep is described. Guidance concerning potential confounding variable are discussed, as are tips for reducing variably among testing sessions.

Key Words: Thermal; nociception; pain; c-fibers; rodents; dogs; hot plate; Hargreaves; skin twitch.

1. Introduction

The acute application of a high-intensity thermal stimulus to the skin is one of the most commonly used models to assess nociceptive processing as an assay to screen for the analgesic activity of a drug or physiological manipulation. In principle, the manipulation serves to activate high-threshold sensory fibers that innervate the skin. These axons transduce temperatures that are in the range of those that produce escape behavior when applied to the skin, with the frequency of discharge proportional to the intensity of the stimulus to which the skin is exposed (1). Such afferent traffic correspondingly activates dorsal-horn neurons located in the superficial spinal lamina, which project in the contralateral ventrolateral tracts to supraspinal sites where they serve to activate neurons in the medulla, mesencephalon, and thalamus (2). The application of such stimuli
evokes a behavioral response that displays several defining characteristics: (1) the escape response evoked by such a focal noxious stimulus is organized somatotopically, e.g., a stimulus applied to the foot will initiate a withdrawal of that foot; and (2) the response latency varies inversely with the intensity of the stimulus \((3,4)\). Drugs or physiological manipulations that diminish the frequency of the evoked discharge or diminish the activity of spinal neurons activated by that input will accordingly increase the response latency \((5)\). If the experimental treatment has no effect on motor function and an increase in latency is observed, then the treatment is said to be antinociceptive or analgesic. Conversely, if a treatment, such as an inflammation of the paw, serves to decrease the response latency, it is said to induce hyperalgesia \((6)\).

The assessment of the acute response of the animal to a strong thermal stimulus is typically accomplished by allowing the animal to stand on a heated metal plate (hot-plate test) \((7)\), placing a heated probe on the skin \((8)\), or placing the tail in heated water (tail-flick immersion test) \((9)\). More recently, these tests have been adapted in which an indirect thermal stimulus is applied to the paw using a heat source such as a focused high-intensity light bulb \((10)\). In this chapter, we will specifically describe the use of a paw thermal escape evoked by: (1) placing the animal on a thermally regulated surface (hot plate); and (2) application of a radiant stimulus to the paw, referred to as the Hargreaves test, both of which are suitable for rodents (mice and rats). In addition, we will describe a skin-twitch reflex test applicable to large animals that was initially performed in spinalized dogs \((8,11,12)\). Protocols for both the hot plate and Hargreaves tests herein will be presented for rats, and experimental adjustments for differences with other species such as mice will be included as appropriate (see Note 1).

2. Materials

2.1. Hot Plate Test

1. Animals: 150–400 g rats. As in all behavioral testing models, animals should undergo several episodes of adaptation to handling and exposure to the test environment. This includes transporting the animal to the test room, placing the animal in the test device, and returning the animal to the home cage.

2. Testing Chamber: Hot-plate apparatus consisting of a Plexiglass enclosure without a bottom placed on the surface of a thermally controlled metal plate as seen in Fig. 1.

a. The plexiglass enclosure is typically about 30 cm high and 15 cm in diameter. These chamber sizes are approximate and are suitable for animals of approx 250 g. Smaller chamber sizes may be employed for proportionately smaller animals. The chamber size should be selected to provide just enough room for the animal to assume a normal crouching posture and sufficient height to prevent the animal to readily escape by jumping. The thermal surface must be of a size that extends beyond the edge of the plexiglass chamber. It must provide for a temperature that is uniform across its surface from edge
Fig. 1. Picture showing a hot-plate system. The metal water bath is placed on top of a stirring hot plate, which has been modified to have a proportional feedback circuit driven by a temperature probe affixed to the metal surface, which constitutes the floor of the thermal escape chamber. A stirring bar is mounted inside of the water bath to ensure uniform mixing and the side filling tube is present to ensure that the chamber is always full to prevent air bubbles inside the water bath.
to edge and be able to maintain the nominal surface temperature to within 1% of the target temperature.
b. Units often employ a water bath with a metal (testing) surface that possesses the desired controlling parameters. The water bath under the testing surface typically requires active circulation of the water bath to avoid hot spots. The temperature control must be governed by the actual measured temperature of the hot-plate surface upon which the animal will be placed.
c. Various commercial hot-plate apparatuses are available, typically offering a range of automation including built in timers and digital output (Columbus Instruments, Columbus, OH; San Diego Instruments, Inc., San Diego, CA; TSE, Technical & Scientific Equipment GmbH, Bad Homburg, Germany).
d. Test environment should be selected as to minimize extraneous noise and traffic. The area should avoid direct drafts from air conditioning or direct sunlight. Mice and rat testing should be carried out in separate rooms to minimize the presence of species-specific odors.

3. 1-cc Syringe with 26 G needle for subcutaneous (sc) or intraperitoneal (ip) drug delivery.
4. Stopwatch or timer for assessing response latency to the nearest 0.1 s.
5. Test article, plus vehicle if appropriate.

2.2. Hargreaves Test

1. Animals: 150–400 g rats.
2. Testing chamber: Thermal nociception testing devices derived from the description by Hargreaves et al. (10) are commercially available from a number of sources. The system shown in Fig. 2 is available from University of California-San Diego (Department of Anesthesiology, Attn. Mr. George Ozaki, 9500 Gilman drive, La Jolla CA 92093-0818). A similar device is also available from Ugo Basile (Biological Research Apparatus, Via G. Borghi 43, 21025 Comerio VA, Italy).
3. Test environment issues should be considered as noted for the hot-plate model.
4. 1-cc Syringe with 26 G needle for sc or ip drug delivery.
5. Stopwatch/timer.
6. Test article, plus vehicle.

2.3. Thermally Evoked Skin Twitch

1. Animals: Purpose-bred beagle dogs (8–16 kg).
2. Testing apparatus: Thermally conductive brass probe with an area of approx 1.2 cm² connected to a heated circulating water bath at 62.5°C. See Fig. 3 for a typical skin-twitch testing unit including an automated timer.
3. Sling for immobilization during testing.
4. 1–3 cc Syringe with 23 G needle for sc or intramuscular (im) drug delivery.
5. Stopwatch or timer.
6. Test article, plus vehicle if appropriate.
3. Methods

3.1. Hot-Plate Test

1. Preheat the hot plate to the selected target temperature, typically 52.5°C (55°C for mice). The targeted temperature should be additionally measured periodically (at the beginning of each day) with a calibrated standard. Records of stability should be maintained with the daily experimental log.

2. For testing, the animal is placed on the hot plate and the timer is started immediately. On the observation of the criterion behavior (see Note 2), the timer is stopped and the animal is immediately removed from the testing apparatus and placed back in its cage. If the animal does not respond within a criterion time (“cut-off time,” see Note 2b), the animal is removed from the testing apparatus to prevent tissue injury and is assigned the maximal cutoff time. Following each test, the surface of the hot plate should be cleansed of any urine and feces.

3. Test sequence typically involves one or two pretreatment baseline response measures at approx 15-min intervals (T = −30, −15 min), delivery of the test article

![Fig. 2. A Hargreaves-type device prepared to permit concurrent testing of six rats. Note the presence of a Voltmeter on the instrument used for tracking changes in stimulus intensity over time.](image-url)
(T = 0) and then testing at preselected intervals thereafter. A typical test time interval would be T = +1, 30, 60, 120, and 180 min. Particular timing will depend on the anticipated time of onset and duration of the test article. See Notes 3–5 concerning data collection and analysis.

4. Suggested drug volume and pretreatment times are listed in Table 1.
3.2. Hargreaves Test Using a Thermal Paw Stimulator

1. The use of radiant-heat devices requires daily assessment of thermal intensity. Different devices may require different protocols. Typically, it will involve two components: (i) Checking the current and voltage delivered to the heating lamp; and (ii) checking the temperature heating curve of the stimulus using a standard temperature probe (thermister or thermocouple) that has a rapid response time (typically T_{1/2} of < 2 s).
   a. As the lamp output will diminish with age, these assessments should be logged daily for apparent shifts over time. Practically, the calibration of the system in the beginning will be accomplished by defining the response latencies in different groups of animals with different stimulus currents and correlating this with the measured heating curve.
   b. Different stimulus systems will display different heating curves vs current relationships and the initial calibration with naïve groups of animals is critical.
   c. Some devices can readily and repeatably alter stimulus currents and hence heating curves. Having multiple stimulus intensities are useful for reliably producing different response latencies by adjusting stimulus intensities.

2. Animals should be acclimatized to the testing apparatus on the day of testing for approx 30 min. Placing a paper towel underneath the animal during this time prevents urine and feces from accumulating on the glass surface. It is important to keep the surface of the glass clean and dry because if water is present on the surface, the thermal transfer characteristics to the paw will be altered.

3. After acclimatization has occurred, rats will display some exploratory behavior such as rearing. At this time remove the paper towels by sliding them from underneath the rat. They will be disturbed briefly but will quickly stop exploratory behavior. Baseline latencies are obtained by moving the light source under the plantar surface of the hind paw as shown in Figs. 4 and 5. The stimulus lamp and timer are simultaneously activated by pressing the Start button. Note 6 discusses response analysis and reporting.

4. Test sequence typically involves one or two pretreatment baseline response measures at approx 15-min intervals (T = –30, –15 min), delivery of the test article

<table>
<thead>
<tr>
<th>Route</th>
<th>Suggested Volumes</th>
<th>Pretreatment Time</th>
</tr>
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<tbody>
<tr>
<td>Oral</td>
<td>10 mL/kg</td>
<td>30 min</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>1 mL/kg</td>
<td>15–30 min</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>4 mL/kg</td>
<td>15–30 min</td>
</tr>
<tr>
<td>Intravenous</td>
<td>1 mL/kg</td>
<td>10 min</td>
</tr>
<tr>
<td>Intrathecal</td>
<td>10 µL + 10 µL flush</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Table 1: Guideline for Volumes and Pretreatment Times for Drug Dosing in the Hot-Plate and Hargreaves Tests in Rats
Fig. 4. A rat in a modified Hargreaves box (UCSD Anesthesiology). The plantar surface of the rear paw is visible in the mirror used to position the focused light source.

Fig. 5. The area represented by the black circle is the target for the focused light beam in the Hargreaves test. Inconsistent placement of the light beam will introduce variability in the results owing to differences in skin thickness throughout the paw.
(T = 0), and then testing at preselected intervals thereafter. A typical test time interval would be T = +15, 30, 60, 120, and 180 min. Particular timing will depend on the anticipated time of onset and duration of the test article. See Notes 3–5 concerning data collection and analysis.

5. If the animal does not respond within a criterion time (“cut-off time”; see Note 6b), the animal is removed from the testing apparatus to prevent tissue injury and is assigned the maximal cutoff time. Following each test, the surface of the testing box should be cleansed of any urine and feces.

6. Animals are returned to their cages on an hourly basis to permit feeding and drinking. They are returned to the test chamber 15 min prior to testing. Re-acclimatization for the 120- and 180-min testing points should require less than 15 min.

3.3. Skin-Twitch Latency in Beagle Dogs

1. Dogs are placed in a sling of appropriate size to minimize movement. It is convenient to place the sling on a table so that the people testing the animal can test in a standing posture. Significant physical restraint is not usually necessary once dogs have been acclimated to the handling, the sling, and testing environment.

2. An area of approx 2 × 3 cm is shaved bilaterally in the lower dorsal T12-L3 region, just off midline and rostral to a line drawn between the pelvic crests.

3. The probe is gently placed within the shaved area on one side until a brief “twitch” is seen localized to the area of probe placement. The probe should be removed immediately following visualization of the response and the latency time is recorded. The maximal time of contact between the probe and skin is 6 s to prevent thermal damage to the skin. If no twitch is present within this time-frame, the probe is removed and a latency of 6 s is recorded. The testing is then repeated on the contralateral side. The sequence is generally repeated only three times per testing interval to prevent tissue damage. Note that different regions within the shaved area should be used for testing so that repeated heating of a small area is avoided. The lowest latency times for the left and right side are averaged to provide a single latency for each time point. See Note 7 for further tips concerning maximizing skin-twitch probability.

4. The observed response is a brisk local contraction of the underlying cutaneous musculature within approx 3 s. On occasion, a dog may display a similar brisk response almost contiguously with the touching of the probe (e.g., latency < 1 s). This is a confounder that reflects the activation of low-threshold, tactile afferents (also referred to as a “flea or fly flick”).

5. As with other thermal testing protocols, the predetermined testing intervals are chosen on the basis of the anticipated pharmacokinetics of the test agent.

4. Notes

1. In the United States, animal studies are undertaken with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). In the absence of other surgical or treatment requirement, these acute stimulation models are considered to
be minimally intrusive or stressful. As described, the animal has complete control over the application of the stimulus. Withdrawal or escape will terminate the stimulus. Comparable studies involving acute escape are routinely performed in humans for somatosensory testing procedures and are uniformly considered to be ethical (13).

2. Hot Plate.

a. Selection of criterion response. Observation of induced behavior after placing the rat on the thermal surface reveals one or more behaviors appearing in the following sequence: (i) grooming with the fore paws; (ii) an increase in ambulatory activity; (iii) rapidly repeated elevations of either hind paw (“foot stomping”); (iv) licking of the hind paw; (v) agitated behavior; and (vi) jumping. Convention has typically focused on the licking of the hind paw to indicate the criterion response. On occasion, an animal will not lick, but show either jumping or evident agitation. It is important that the same end point be used throughout the study. However, if jumping is seen, that must be taken as an endpoint because clearly it is evidence of escape. Similarly, a high level of agitation (frantic ambulation around the hot-plate surface or vocalization), although less desirable and definitive, must also be taken as a termination endpoint. In these cases where an alternate endpoint is employed, that should be part of the data record (e.g., 12J or 12A, indicating jump or agitation at 12 s as the endpoint).

b. Selection of criterion cut-off time. The purpose of the cutoff time is to prevent significant injury. As the test-surface temperature is increased, typically the shorter the baseline response latency and the shorter the time the animal can be allowed to remain on the surface without paw injury (oedema, erythema, or blistering). It is important to avoid injury with repeated testing to avoid creating a sensitized skin surface that will confound drug effects. In general, with a 52.5 ± 0.5°C surface, repeated testing may be carried out by convention with a 40-s response cut-off for a rat and 30-s response cut-off for a mouse. Selection of a cut-off time should be undertaken as part of the calibration of any test system.

c. Criterion response behaviors. Determining which one of the possible behaviors will be quantified, e.g., the paw lick or jump, should be done prior to testing; this behavior should be used consistently throughout the study. Selection of the behavior may be influenced by drug side effects; for example, morphine increases motor activity in mice, thus paw lick would be a more appropriate measure.

3. Data for hot plate, Hargreaves test, and thermal skin twitch may be expressed as mean and SEM of the response latency in seconds. Alternately, these data may be expressed as percent maximum possible effect (%MPE). This is calculated using the formula below

\[\text{%MPE} = [(\text{latency}–\text{baseline})/(\text{cutoff}–\text{baseline})] \times 100\]

4. The use of the MPE has several advantages. (a) It allows the researcher to normalize the results for each animal with respect to its own control. It is assumed that the baseline differences between animals in a given treatment are distributed randomly...
and this normalization procedure will reduce between-animal variations. On the other hand, if there are systematic differences in baseline (secondary to other treatments, strain differences, or differences in the experimental apparatus calibrations), then the normalization procedure will be essentially misleading. In such cases, raw response latencies should be part of the discussion of the data set. (b) If one has generated a dose-response curve using several doses, the dose of the test article that produces a 50% maximal effect (ED$_{50}$) can be calculated to compare the relative potency of different drugs.

5. Careful and systematic assessment of motor and sensory function using either a functional observation battery (FOB) is suggested when testing drugs that may alter motor function or may be either sedative or stimulants. For example, a rat treated with a neuroleptic such as the dopamine D2 antagonist haloperidol or with high doses of stimulants such as amphetamine may be unable to lick the hind paw or jump when placed on the hot plate, but this does not mean that these compounds have analgesic efficacy. These drugs have simply disrupted motor function by causing akinesia in the case of the haloperidol or stereotypy in the case of amphetamine.

6. Hargreaves model
   a. The criterion response is the abrupt withdrawal of the stimulated hind paw. Upon withdrawal of the paw, the stimulus and timer are terminated. The latency time is recorded and the opposite hind paw is similarly tested. If either hind paw is normal, then the response latencies for the two paws are averaged to obtain a single value for each paw.
   b. Selection of criterion cut-off time. As described for the hot plate, the purpose of the cutoff time is to prevent significant injury. It is important to avoid injury with repeated testing to avoid creating a sensitized skin surface, which will confound drug effects. Selection of a cutoff time should be undertaken as part of the calibration of any test system. Usually, a 20-s cut-off time is chosen for a rat and a 15-s cut-off time is chosen for a mouse.
   c. The above description has been for the assessment of acute nociception. The model is expanded readily to consider the effects of treatments that serve to produce hyperalgesia. For example, injection of an irritant such as carrageenan ([λ]-Carrageenan, Sigma Chemical Company, St. Louis, MO) into the dorsum of the paw will result in a progressive appearance of inflammation over 2–3 h. The paw becomes edematous and swollen. Testing the thermal escape latency of the inflamed paw will show that its response latencies are much reduced as compared with the noninjected paw (14,15). This enhanced behavioral responsiveness reflects both a local peripheral sensitization, such that the afferent fires vigorously in response to a modest stimulus, and a central sensitization, in which there is an exaggerated response to afferent input (16,17).

7. Thermal Skin Twitch.
   a. It should be noted that there can be significant variability between dogs with latencies, ranging from 1 s to greater than 6 s. In general, with a 62.5°C probe, repeated testing may be carried out by convention with a 6-s response cut-off. It is our routine practice to screen dogs and exclude animals with latency greater
than 6 s after three acclimatization sessions. Additionally, it is important to verify that the twitch truly is thermally evoked, and not owing to tactile cues or hypersensitivity. This can be accomplished easily by applying an equally sized and thermally neutral stimulus, such as an unsharpened pencil, to the testing area.

b. Dogs are allowed to acclimatize for at least three testing sessions prior to collection of baseline data. Most dogs readily acclimatize to standing in the sling for the duration necessary for nociceptive testing. When anxious, some dogs will often display a very minor whole body shaking, during which time the twitch reflex is inhibited.

Thermal nociception assays of some sort are a common feature of many experimental laboratories studying the perception of pain, including both laboratory animals and humans subjects. They provide rapid and reliable models to assess the effects of physical or pharmacological treatments on the perception of acute pain.

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