Differentiating Thermal Allodynia and Hyperalgesia Using Dynamic Hot and Cold Plate in Rodents

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Abstract: In animal studies, thermal sensitivity is mostly evaluated on the basis of nociceptive reaction latencies in response to a given thermal aversive stimulus. However, these techniques may be inappropriate to differentiate allodynia from hyperalgesia or to provide information differentiating the activation of nociceptor subtypes. The recent development of dynamic hot and cold plates, allowing computer-controlled ramps of temperature, may be useful for such measures. In this study, we characterized their interest for studying thermal nociception in freely moving mice and rats. We showed that escape behavior (jumps) was the most appropriate parameter in C57BL/6J mice, whereas nociceptive response was estimated by using the sum of paw lickings and withdrawals in Sprague-Dawley rats. We then demonstrated that this procedure allows the detection of both thermal allodynia and hyperalgesia after peripheral pain sensitization with capsaicin in mice and in rats. In a condition of carrageenan-induced paw inflammation, we observed the previously described thermal hyperalgesia, but we also revealed that rats exhibit a clear thermal allodynia to a cold or a hot stimulus. These results demonstrate the interest of the dynamic hot and cold plate to study thermal nociception, and more particularly to study both thermal allodynia and hyperalgesia within a single paradigm in awake and freely moving rodents.

Perspective: Despite its clinical relevance, thermal allodynia is rarely studied by researchers working on animal models. As shown after stimulation of capsaicin-sensitive fibers or during inflammatory pain, the dynamic hot and cold plate validated in the present study provides a useful tool to distinguish between thermal allodynia and thermal hyperalgesia in rodents.

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Key words: Pain, dynamic hot/cold plate, hyperalgesia, allodynia, capsaicin, carrageenan.

Thermnociception in rodents is usually evaluated by measuring escape latency in response to a nociceptive stimulus at fixed temperature. Tail-flick and the hot plate are widely used to assess responses to heat stimuli. These tests have been used by researchers for over half a century; they are well standardized and considered as references for testing analgesic drugs. In the late 1980s, the development of the Har

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known to be nociceptive, that is, hyperalgesia. Despite its relevance to clinical conditions, thermal allodynia is more rarely considered in animal studies.

The objective of our study was to establish a reliable method to score mouse and rat nociceptive behaviors using computer-controlled ramps of temperature and to validate this method in animals with pain symptoms. Using a dynamic hot and cold plate analgesia meter, we identified the most relevant behavioral parameters in C57Bl/6J mice and in Sprague-Dawley rats and we evaluated the responses to the sensitization of capsaicin-sensitive C-fibers or to carrageenan-induced hind paw inflammation. Our results reveal that the dynamic hot and cold plate allows the measurement of both thermal alldynia and thermal hyperalgesia within the same experimental procedure.

Materials and Methods

Animals

Male C57Bl/6J mice (weight, 25 to 30 g; Charles River, L’Arbresle, France) and Sprague-Dawley rats (weight, 250 to 350 g; Janvier, Le Genest St Isle, France) were used for the experiments. Mice and rats were housed 4 per cage in independent animal rooms. They were maintained under a 12-hours light/dark cycle in standard animal housing conditions, with food and water ad libitum. All animals were habituated to the apparatus before the onset of the experiments. All procedures were performed in accordance with European directives (86/609/EEC) and were approved by the regional ethics committee and French ministry of agriculture (license No. 67-116, to P.P.).

Tests

For the hot or cold plate tests, we used a computer-controlled hot and cold plate analgesia meter (Bioseb, Chaville, France). With no animal on it, the plate can be heated up to 65°C or cooled down to –3°C ± 0.1°C in an ambient room temperature (20°C to 25°C). The animal was placed in a Plexiglas cylinder (mice: 10-cm diameter, 15-cm height; rats: 15.5-cm diameter, 25-cm height) with a drilled cover.

Conventional Hot Plate Test

Mice or rats were placed on the hot plate with the temperature adjusted to 52°C ± 0.1°C (n = 5/group for mice; n = 6/group for rats) or to 42°C ± 0.1°C (n = 5/group for mice; n = 4/group for rats). The latency to the first hind paw licking or withdrawal was taken as an index of nociceptive threshold. The cutoff time was set at 15 seconds and 60 seconds, respectively, to avoid damage to the paw.

Dynamic Hot Plate Test

Animals were placed on the dynamic hot plate (DHP) at 30°C ± 0.1°C, and the plate temperature increased up to 43°C for mice (n = 10 for the characterization of DHP, for dimethylsulfoxide (DMSO) and for capsaicin, n = 5 for the respective control rats with 0.9% NaCl or 50% ethanol in saline) or 45°C for rats (n = 8 for the characterization of DHP, n = 4 for DMSO, capsaicin and control rats: 0.9% NaCl or 50% ethanol in saline), with 1°C.min⁻¹ speed (r² = 1). During each degree interval, we scored hind paw lickings, escape behavior (jumps), and rearing for mice; hind paw lickings, paw withdrawals, and rearing for rats.

Dynamic Cold Plate Test

Animals were placed on the dynamic cold plate (DCP) at 20°C ± 0.1°C, and the plate temperature was decreased down to 1°C for mice (n = 10 for the characterization of DHP, n = 6 for DMSO, n = 7 for capsaicin, n = 8 for the respective control rats with 0.9% NaCl and 50% ethanol in saline) or 5°C for rats (n = 8 for the characterization of DHP, n = 4 for DMSO, for capsaicin and control rats: 0.9% NaCl or 50% ethanol in saline), with a 1°C.min⁻¹ speed (r² = 0.99). Scored behaviors were determined as described with DHP.

Hargreaves Test

Mice (n = 5/group) and rats (n = 6 for DMSO, capsaicin or control rats with 50% ethanol in saline; n = 8 for control with 0.9% NaCl) were placed in clear Plexiglas boxes (mice: 7 cm × 9 cm × 7 cm; rats: 22 cm × 19 cm × 14 cm) on a glass surface, and testing began after exploration and grooming behaviors ended (15 minutes). The infrared beam of the radiant heat source (7370 Plantar Test; Ugo Basile, Comerio, Italy) was applied to the plantar surface of each hind paw. The experimental cutoff to prevent damage to the skin was set at 15 seconds. Three measures of the paw withdrawal latency were taken and averaged for each hind paw.

Peripheral Sensitization

We studied the effect of local application of capsaicin or DMSO in mice and rats. Capsaicin (10 mM, dissolved in 50% ethanol with 0.9% NaCl) or DMSO (100%) was applied topically on the plantar surface of the mice or rat hind paws with cotton-tipped applicators. They were tested 15 minutes after the application of the drugs. As a control, we also tested mice or rats at fixed temperature (30°C) for 15 minutes after a topical application of saline, capsaicin, or DMSO.

In rats, we determined the consequence of carrageenan-induced inflammation using the dynamic hot and cold plate test. The λ-carrageenan (3% in 0.9% NaCl, 150 μL) was injected in the right hind paw of the rats. We only evaluated the effects of carrageenan-induced inflammation in rats because this model was well characterized in rats, and few studies exist in mice. Injections and applications (all drugs from Sigma-Aldrich, St. Quentin Fallavier, France) were done under light 3% halothane anesthesia. Control groups received the vehicle of the administered drug.

Statistics

The data are expressed as mean ± SEM. Because data did not fit the conditions of parametric testing (homogeneity of variances and normality of samples), nonparametric tests
were used. Statistical analyses were performed with STATISTICA 7.1 (Stat-soft, Tulsa, OK). The independent variables were analyzed by Kruskal-Wallis $H$ tests followed by Mann-Whitney $U$ test when significant differences were detected. The application of capsaicin or carrageenan was taken as independent variable (capsaicin or carrageenan vs control). The Wilcoxon test for 2-group comparisons or multiple-factor analysis of variance (ANOVA), Friedman & Concordance of Kendal, was used when the variables were dependent. The behavioral changes due to temperature were taken as dependent variables because the repeated measures were done on the same animal. The significance level was set at $P < .05$.

**Results**

**Nociceptive Parameters in C57Bl/6J Mice**

During DCP testing in mice (Fig 1A), we observed no major change in paw licking or rearing behaviors, but

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**Figure 1.** Dynamic cold (DCP) and hot-plate (DHP) tests in mice. A, Numbers (nb) of rearing, hind paw licking, and jumps were measured on the DCP from 20°C to 1°C. B, The same parameters were measured on the DHP from 30°C to 43°C. The tests were done on C57Bl/6j mice (n = 10/group). The cooling and heating rate for the dynamic cold and hot plate was 1°C min$^{-1}$. Statistical significance for jumps is indicated (*$P < .05$, **$P < .01$, when compared with the response at starting temperatures).

**Figure 2.** Peripheral sensitization in mice. A, Jumps after topical application of DMSO or vehicle (control: 0.9% NaCl) in the DCP test (DMSO, n = 6; control, n = 8). B, Jumps after topical application of DMSO or vehicle (control: 0.9% NaCl) in the DHP test (DMSO, n = 10; control, n = 5). C, Paw licking latency after topical application of DMSO or vehicle in the conventional hot plate test (setup at 52°C or at 42°C) or in the Hargreaves test (n = 5/group). D, Jumps after topical application of capsaicin or vehicle (50% ethanol in saline) in the DCP test (capsaicin, n = 7; control, n = 8). E, Jumps after topical application of capsaicin or vehicle (50% ethanol in saline) in the DHP test (capsaicin, n = 10; control, n = 5). F, Paw licking latency after topical application of capsaicin or vehicle in the conventional hot plate test (setup at 52°C or at 42°C) or in the Hargreaves test (n = 5/group). The cooling and heating rate for the dynamic cold and hot plate was 1°C min$^{-1}$. Statistical significance against the respective control animals is indicated (*$P < .05$, **$P < .01$). y-axes give the number (nb) of responses.
Peripheral Sensitization in C57Bl/6J Mice

DMSO application did not alter responses in the DCP (Fig 2A), in the DHP (Fig 2B), or in the conventional hot plate test at 52°C and 42°C, and in the Hargreaves test (Fig 2C). Topical application of capsaicin significantly augmented the number of jumps from 19°C in the DCP test ($P < .05$; Fig 2D). It also decreased temperature threshold for jumps (from 39°C to 35°C) in the DHP ($P < .05$ for 35°C and above; Fig 2E). The number of jumps was significantly increased for temperatures above 39°C ($P < .05$). No jump was observed after capsaicin application when mice were tested for 15 minutes on the plate setup at 30°C (data not shown).

Capsaicin did not alter the conventional hot plate response at 52°C, but it decreased licking latency at 42°C ($P < .01$) or in the Hargreaves test ($P < .05$) (Fig 2F).

Nociceptive Parameters in Sprague-Dawley Rats

During DCP tests in rats (Fig 3A), a significant increase in rearing behavior ($H = 80.71, P < .001$), hind paw licking ($H = 69.69, P < .001$), and withdrawal ($H = 105.74, P < .001$) was seen at 9°C. In the DHP tests (Fig 3B) and for temperature above 39°C, we found significantly elevated scores for rearing behavior ($H = 51.84, P < .001$), hind paw licking ($H = 58.71, P < .001$), and withdrawal ($H = 91.46, P < .001$). The sum of paw lickings and withdrawals was used as index of nociceptive response (DHP: $H = 101.39, P < .001$; DCP: $H = 112.85, P < .001$) (Fig 3, C and D). The temperature inducing the first significant withdrawal reaction was of 9.31°C ± 0.15°C with DCP (Fig 3E) and of 39.58°C ± 0.21°C with DHP (Fig 3F). This value was not affected when the test was repeated (Fig 3, E and F) over a period of 10 days. No escape behavior was observed when rats were tested for 15 minutes at 30°C or 20°C (data not shown).

Peripheral Sensitization in Sprague-Dawley Rats

DMSO application did not modify nociceptive responses in the DCP (Fig 4A), in the DHP (Fig 4B), in the conventional HP at 52°C and 42°C, and in the Hargreaves test (Fig 4C) in rats. Topical application of capsaicin significantly augmented the number of jumps between 11°C and 9°C ($P < .05$; Fig 4D) in the DCP test. It also decreased temperature threshold for jumps (from 40°C to 34°C) in the DHP ($P < .05$ for 34°C and above; Fig 4E). Capsaicin did not alter hot plate response at 52°C or in the Hargreaves test but it decreased licking latency in the conventional hot plate at 42°C; $P < .05$ (Fig 4F).

Carrageenan-Induced Inflammation in Sprague-Dawley Rats

Carrageenan-injected rats displayed significant nociceptive response in DCP (Fig 5A) at 13°C instead of 9°C for control rats ($P < .05$) and in DHP (Fig 5B) at 37°C instead of 39°C ($P < .05$). In addition to cold and heat allodynia, thermal hyperalgesia in carrageenan-injected
Discussion

In this study, we characterized the use of the DCP/DHP test to measure nociceptive responses to thermal stimuli in C57Bl/6J mice and in Sprague-Dawley rats. In each species, we identified the behavioral responses that are the most relevant for nociceptive measure. This method was further validated by using 2 models of pain sensitization including capsaicin-induced sensitization of peripheral nociceptors in mice and in rats and carrageenan-induced peripheral inflammation in rats. Our results showed that the DCP/DHP test can reveal both thermal cold/hot alldynia and hyperalgesia within a single procedure in awake and freely moving animals.

C57Bl/6J mice and Sprague-Dawley rats exhibit different responses in the DCP/DHP test. In both species, rearing behavior that reflects spontaneous activity and exploration did not appear suitable to measure nociceptive response. Jumps, which can be interpreted as an escape behavior, were only present in mice when hot- or cold-nociceptive temperatures were reached and the number of jumps increased with the intensity of nociceptive stimulus. It is thus the most relevant parameter to score during DCP/DHP tests in the C57Bl/6J mice. Similarly, hind paw lickings and withdrawals appeared as the most relevant parameters in Sprague-Dawley rats.

Cumulating both parameters in rats gave a clear-cut detection of the nociceptive response and threshold. When we tested mice or rats during 15 minutes on the plate setup at 30°C or 20°C, we did not observe any nociceptive response was indicated by increased nociceptive responses at 9°C or 45°C (P < .05).
behavior. Moreover, the procedure offers reproducible measures as shown by repeated testing on a 9-day period.

The nociceptive threshold to heat in the DCP/DHP test was between 39°C and 40°C. This is in agreement with thermonociceptor thresholds as measured by in vivo electrophysiology. However, there is no clear agreement in the literature as to when a cold stimulus begins to be nociceptive. The results that we obtained with the cold ramp revealed a behavioral response starting at 4°C for mice and at 9°C for rats. These temperatures could be considered as nociceptive because the animals did not display escape behavior (mice) or paw licking/withdrawal (rats) before reaching them.

Thermal nociception is thought to be mediated by C- and Aδ-fibers, which have distinct electrophysiological and pharmacological properties. Based on in vivo electrophysiological approaches, it has been proposed that slow ramps (speed) of temperature heating preferentially activate C-fibers, whereas fast ramps (speed) of temperature heating preferentially activate Aδ-fibers. This idea is also supported by experiments using contact heating or infrared diode laser systems to distinguish thermonociceptors. Thus, the 1°C/min heating rate that we used in this study can be considered as very slow when compared with other studies, and we suggest that this slow rate heating preferentially activates C-fibers. Unfortunately, we have no direct evidence such as peripheral nerve recordings. Moreover, for doses similar to the ones we used, capsaicin and DMSO were proposed to act respectively on C- or Aδ-thermonociceptors. Accordingly, we found significant heat hypersensitivity with topical application of capsaicin in mice and rats with DHP testing. However, we observed no effect of topical DMSO application either in DHP or DCP testing in both mice and rats. Our data indicating that C- or Aδ-thermonociceptors could be differentially activated depending of the rate of skin heating are in good agreement with the results of the groups of Lumb and Yeomans, and a main interest of the DCP/DCP test was to provide a procedure to study this sensitization of thermonociceptors in freely moving animals. Interestingly, we observed that capsaicin-treated mice and rats showed nociceptive behavior at innocuous temperatures, which revealed the presence of a thermal allodynia.

On the other hand, the conventional HP setup at 52°C did not allow the detection of an effect of either capsaicin or DMSO application in mice and rats. The Hargreaves test and the conventional hot plate at 42°C with a long cutoff allowed detection of the capsaicin effect in mice but did not discriminate between thermal alldynia and thermal hyperalgesia. On the contrary, in rats we observed the effects of capsaicin with the conventional hot plate at 42°C but not with the Hargreaves test. The DCP/DHP is thus of interest to provide both within the same testing procedure.

Similarly, we investigated whether this method is able to measure changes in hypersensitivity evoked by a carrageenan-induced inflammatory pain model. Because this model is first and well characterized in rats, we decided to evaluate the effects of carrageenan-induced inflammatory pain in DCP/DHP testing in rats. We observed that carrageenan-injected rats display a nociceptive reaction at innocuous temperatures (ie, thermal allodynia) as well as an exaggerated number of responses at noxious temperatures (ie, thermal hyperalgesia) in comparison to saline-injected rats. These data revealed that inflammatory pain is inducing a strong thermal allodynia and confirmed in a widely used pain model the interest of the DCP/DHP procedure to observe thermal allodynia as well as hyperalgesia in the same procedure.

We observed that capsaicin-treated mice and rats or carrageenan-injected rats display hyperalgesia and/or allodynia as compared with their control animals. Although the DCP/DHP requires longer testing time than other thermal tests, it allows measuring the nociceptive temperature thresholds and behavioral score for nonnoxious and noxious stimuli in a single experiment with a freely moving animal.

References


16. Lawson SN: Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 87:239-244, 2002


32. Zachariou V, Goldstein BD, Yeomans DC: Low but not high rate noxious radiant skin heating evokes a capsaicin-sensitive increase in spinal cord dorsal horn release of substance P. Brain Res 752:143-150, 1997