Calibrated Forceps: A Sensitive and Reliable Tool for Pain and Analgesia Studies

Oliva Erendira Luis-Delgado, Michel Barrot, Jean-Luc Rodeau, Grégory Schott, Malika Benbouzid, Pierrick Poisbeau, Marie-José Freund-Mercier, and François Lasbennes

Institut de Neurosciences Cellulaires et Intégratives (INCI), UMR7519 CNRS/Université Louis Pasteur, Strasbourg, France.

Abstract: Devices designed for mechanical pain threshold studies are often difficult to implement. The purpose of this study was to investigate a simple tool based on calibrated forceps to induce quantifiable mechanical stimulation in the rat on a linear scale. The most suitable protocol was tested by determining the effects of 3 repetitive measurements on both hind paws, respectively, during long-term (9 days), mid-term (1 day), and short-term (2 hours). Only threshold increase related to weight gain over long-term was observed, suggesting that moderate rat training can be used. The capacity of the device to reveal hyperalgesia was tested in a model of carrageenan-induced inflammation in the hind paw. The hyperalgesia was maximal 6 hours after carrageenan injection and progressively decreased. Similar, although more variable, responses were observed with von Frey filaments. Morphine-induced analgesia resulted in a dose-dependent increase of paw threshold. Tolerance to morphine administrated on a once daily schedule (10 mg/kg) during 5 days was revealed by a significant decrease in analgesia by day 3. Taken together, these results demonstrated accuracy of this device for easy, fast, and reproducible measure of mechanical pain threshold on rat limbs. Moreover, it allows the performance of rat testing with minimal constraint, which reduces data variability.

Perspective: The calibrated forceps is an easy to use device well-suited to rapidly test mechanical pain threshold with accuracy. It is well-designed for preclinical behavioral screening of noxious or analgesic properties of molecules.

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threshold to a graduated increase of the stimulus. Devices used for the test by Randall and Selitto26 or the modified Randall-Selitto test (analgesy-meter) apply a known progressive pressure on the paw placed between a flat surface and a blunt pointer. Paw withdrawal corresponds to the threshold pressure. The main drawback of this technique is the necessity to restrain the animal in a vertical position to maintain the paw on the flat surface. This factor of stress, which might interfere with pain evaluation, is circumvented by overtraining the animals. Von Frey filaments are another way to evaluate mechanical hyperalgesia.4 A calibrated filament is applied on the paw without restraining the animal. This method, which is useful for assessing allodynia, might have limitations for measures of mild changes in mechanical sensitivity. Indeed, the precision of the measure might be affected by the opposing force performed by the animal limb on the filament and/or by the logarithmic scale of the measure.

Recent studies started to use home-made calibrated forceps, allowing one to progressively apply hand pressure between the tips of the forceps until paw withdrawal or vocalizations.28,31 In the present study we used a standardized device composed of strain gauges glued to the sides of the forceps and connected to an electronic recorder (Bioseb, Chaville, France) that displays the applied force. This device needs only to gently restrain the animal, which is maintained in a natural position, and the force applied between the 2 tips is independent of the movements of the limb. The aim of this study was to evaluate accuracy, sensitivity, and reliability of this device by using carrageenan-induced inflammatory pain and morphine analgesia and tolerance.

Materials and Methods

Animals

Male Sprague-Dawley rats (180 to 200 g; Janvier, Le Genest St Isle, France) were grouped and housed under standard conditions (room temperature, 24°C; 12/12-hour light/dark cycle; lights on at 7 AM) with ad libitum access to food and water. All animals were allowed at least 1-week habituation to the animal room before the experiments started. On the experiment days, the rats were brought to the behavioral testing room 2 hours before testing. Behavioral tests were done during the light phase. All procedures were performed in accordance with the European Communities Council Directive (86/6609/EEC).

Calibrated Forceps

The device (developed and provided by Bioseb) (Fig 1) consists of a pair of large blunt forceps (15 cm long; flat contact area; 7 mm × 1.5 mm with smooth edges) equipped with 2 strain gauges (Fig 1B) connected to a modified electronic dynamometer (Fig 1A). Calibration of the instrument including the 2 branches of the forceps and the dynamometer was performed by Andilog Technologies according to the NF EN 10002-2 European standard instruction of December 1991. Real-time value of the force exerted on the tip of the forceps can be displayed in different units of measure (g, kg, N, mN, oz, and lb). Results of the present study were expressed in g.

The tested rat was placed on the bench and loosely restrained by using a towel masking the eyes to limit influences from environmental stimulations. The tips of the forceps were placed around the hind paw (Fig 1C), and care was taken to apply the same tip length on the hind paw for each trial. The force applied was then incremented by hand at a speed of approximately 200 g every 3 seconds until the paw withdrawal. An analogical display unit on the dynamometer allows the experimenter to train for this device and to check mechanical force level over time during the test. The maximum force applied on the paw was automatically recorded and displayed by the dynamometer. Measurement was repeated 3 times for each hind paw during each testing session, and the mean force exerted
for each paw was determined. Depending on the experiment, this procedure was either repeated on consecutive days or within the same day with 30-minute or 1-hour intervals.

**Von Frey Filaments**

We used Touch Test von Frey filaments (Bioseb) with logarithmic incremental stiffness (4, 6, 8, 10, 15, 26, 60, 100, and 180 g) to determine the nociceptive mechanical threshold of the animals. The paw pressure threshold was defined as the lowest force of the filaments producing at least 4 withdrawal responses out of 5 trials.

**Carrageenan-Induced Inflammation**

The inflammation was induced in the right hind paw by injection of Lambda carrageenan type IV (150 μL, 3% solution in 0.9% NaCl sterile) (Sigma-Aldrich, St Quentin Fallavier, France; lot 80K1334). The injection needle (26-gauge) punctured the plantar skin and was placed in the subcutaneous space just proximal to the foot pads. The same volume of sterile 0.9% NaCl was administered to control animals.

To determine the time course of hyperalgesia and allodynia, changes in mechanical nociceptive thresholds were measured on all rats before and 1, 2, 3, 4, 5, 6, 7, and 24 hours after the 3% carrageenan injection. This was done by using the calibrated forceps (n = 4 per group) and on a separate set of animals by using the von Frey filaments (n = 5 per group). Paw volume was measured at same time points by water displacement. The hind paw of the rat was dipped up to the limit of the heel glabrous surface into a water-filled vial placed on a precision scale balance (sensitivity, 0.1 g; Sartorius, Göttingen, Germany).

**Morphine-Induced Analgesia**

Morphine sulfate (Francopia, Aramon, France; 0, 2.5, or 10 mg/kg, diluted in 0.9% NaCl, n = 4 per group) was given subcutaneously 30 minutes before testing analgesia. For the tolerance experiment (n = 4 per group), the animals received a daily injection of the dose 10 mg/kg. In each of these experiments, the paw pressure threshold was measured both before morphine injection (referred to as pre-injection) and 30 minutes after morphine injection (referred to as post-injection). A cutoff force was fixed at 2000 g to prevent tissue damage; this cutoff was approximately twice the force inducing a response in control conditions.

**Statistical Analysis**

Data are presented as mean ± standard error of the mean. Statistical analysis was performed with Statistica 5.1 (Statsoft, Tulsa, Okla) by using multifactor analysis of variance (ANOVA). Measurement order within a triplicate (1,2,3), side of measurement (left or right paw), or time of measurement were taken as within-subject factors, with repeated measures on the same animal, and the substance injected (saline/carrageenan or morphine) as a between-groups factor; interaction between factors was also tested. The Tukey test was used when appropriate for post hoc multiple comparisons between individual points. The significance level was set at P = .05.

**Results**

**Calibrated Forceps Test: Basal Nociceptive Threshold**

The animals were tested during a period of 9 days to evaluate the effect of repeated measures between days. Before test session the animals were shortly handled on a daily basis for a week to favor manipulation-induced stress decrease. On each test day, the nociceptive threshold values from the 3 consecutive tests on both rat hind paws were measured. Three-way ANOVA did not show any significant difference in pressure threshold between the 3 measures made or between the 2 hind paws (Fig 2A, B); the only significant effect was that of time (F3,52 = 94, P <.001). Further regression analysis confirmed a significant (R = .87, P = .003) linear increase of average threshold values during the 9 days (slope, 29.1 ± 6.2). During the same period rats gain weight (Fig 2B, inset), and a high correlation was observed (R = 0.90, P <.001; slope, 3.36 ± 0.34) between pressure threshold and rat weight.

To evaluate the effect of repeated tests within a day, we measured the nociceptive paw threshold at 30-minute intervals for 2 hours, both in the morning and in the afternoon. Four-way ANOVA did not show any significant difference between the 3 consecutive measures for each triplicate or between left and right paws (Fig 2C); there was no significant change in measurements during each 2-hour session, whether it was AM or PM (Fig 2D). Therefore, the progressive increase in threshold observed in Fig 2A cannot be detected on a shorter time course.

Because no significant difference was found between the 3 consecutive measures for each triplicate, all further analyses were made by using the average value of these 3 pressure measures.

To evaluate reproducibility between investigators, nociceptive paw threshold was measured by 2 experimenters during 4 days on each hind paw of the same rat group (n = 5). Whereas one individual was naive, the other was usually in charge of this test. The 2 independent test sessions were separated by 30 minutes, and users order was permuted every day. Three-way ANOVA did not show any significant difference between users results (Fig 2E).

**Paw Edema After Carrageenan Injection**

After 3% carrageenan injection in right hind paw, the edema reached a maximal volume after 5 to 6 hours (Fig 3A). Three-way ANOVA showed a significant interaction of the 3 factors, treatment, time, and side (F3,48 = 55.0, P < .001). Paw edema became significant at 3 hours and remained close to maximum level 24 hours after injection. No changes in paw volume were observed in the control group or in the contralateral paw (n = 4 rats per group).
Carrageenan-Induced Mechanical Hyperalgesia

Calibrated Forceps Test

We observed a time-course effect of carrageenan-induced inflammation on the mechanical nociception by using the calibrated forceps (Fig 3B). Statistical analysis showed a significant interaction of treatment, time, and side ($F_{8.48} = 6.89, P < .001$). Carrageenan treatment caused a significant decrease in paw threshold response to noxious mechanical stimulation when compared with controls. This decrease was significant at 2 hours, and the minimal threshold value (mechanical allodynia) was reached 6 hours after injection. Mechanical hyperalgesia was still present at 24 hours after injection.
We used the von Frey filaments as a comparison device to the calibrated forceps (Fig 3C). Three-way ANOVA showed significant effects of treatment × side interaction ($F_{1,6} = 8.78, P = .025$) and time × side interaction ($F_{8,48} = 2.80, P = .012$) only, although with 1 more animal ($n = 5$) per group. Mean paw withdrawal threshold was significantly lower in the ipsilateral than in the contralateral paw of carrageenan-injected animals; the decrease was significant at 4 and 5 hours after carrageenan injection; there was no difference in saline-injected ones.

**Analgesic Effects of Morphine**

A dose-dependent analgesic effect of morphine was shown in post-injection tests (Fig 4). The cutoff level (2000 g) was reached for all measures at 10 mg/kg (Fig 4A).

The induction of morphine tolerance was studied by daily subcutaneous administration of 10 mg/kg for 5 days (Fig 4B). Nociceptive threshold values were measured every day just before and 30 minutes after morphine administration. On day 1 the paw pressure threshold reached the 2000-g cutoff. Four-way ANOVA with factors treatment (morphine/saline), time (days 1, 2, 3, 4, and 5), injection (pre/post), and paw (left/right) confirmed the absence of difference between the values for the left and the right paws, which could be expected. A significant effect of the interaction factor treatment × time × injection ($F_{4,24} = 4.82, P = .006$) was observed. Morphine pre-injection values were not different from control values obtained with saline injection and showed no significant difference during the 5 days. The paw nociceptive threshold after morphine injection progressively decreased during the 5 days of treatment (Fig 4B). Values were significantly higher than saline control for the first 3 days (Fig 4B).

**Discussion**

Our study demonstrated that tests with calibrated forceps to measure mechanical sensitivity in rats are reliable, sensitive, and easy to perform.

**Calibrated Forceps Test**

Calibrated forceps were used for different purposes. Their simplest use consisted of applying controlled nerve compression in rats to induce injury. Some other studies used forceps as a means to induce mechanical stimulation during electrophysiologic recording. Recently, they were also used to measure knee joint pain in experimental arthritis and primary hyperalgesia induced in deep tissues by inflammation by directly applying the forceps to the muscle or to the knee joint. However, calibrated forceps were not yet used for systematic measure of nociceptive paw pressure threshold.

This easy to use device allowed us to perform rapid measurements, 3 minutes per animal in the present study. This throughput can probably even be increased by suppressing the triplet measurement at each time point. Indeed our results show that measures are stable within these triplet measurements. Therefore in a time-course study, it should be possible to increase the yield of the protocol without dramatic consequences on precision by performing only 1 measure for each test session.
Moreover, a time-course experiment during a period of 1 day can easily be performed because repeated measurements during the same day showed no significant difference. Furthermore, this device did not need special care like von Frey filaments, which can be affected by temperature and humidity and can be deformed by extensive use.22

Figure 4. Changes of the nociceptive hind paw threshold induced by morphine. (A) Dose-response histograms of morphine-induced analgesia. Two-way repeated-measures ANOVA showed a significant effect of dose \(F_{2, 9} = 118.2, P < .001; n = 4\) rats per dose. \(\ast P < .05, \ast\ast P < .001\) (vs pre-injection value; Tukey test). (B) Time-course effect of once-daily morphine or saline administration (n = 4 rats per treatment) on nociceptive threshold measured before and after injection. Note that the value at day 1 after morphine injection is underestimated because of the experimental cutoff at 2000 g. Linear regression analysis on mean values at days 2 to 5 confirms a significant decrease of analgesia with time \((R = -0.98, P = .015)\). \(\ast\ast\ast P < .001\) (vs pre-injection or saline controls; Tukey test).

The correlation observed during a period of 9 days between the curve expressing weight gain and the threshold curve suggests that the modification of pain sensitivity was due to the increased size of the paw. Because this nociceptive drift is shallow, pain measurements can be compared during periods as short as 3 days during which threshold values did not significantly change. For longer periods it would be preferable to use heavier rats (older) characterized by lower weight gain. The low variability obtained during the 9 days, within each day, or within each test triplet suggests that the gentle restraint of the rat during the test is relatively stress-free. Moreover, the absence of significant difference between the 2 legs, together with the low variability, indicates that calibrated forceps is perfectly suitable for studies with one paw as control for the other.

The study designed to evaluate a possible experi- menter effect showed that values are very similar from one investigator to another, whether he was highly trained or not. It is obviously impossible with this device to reproduce by hand identical mechanical implementations for every test. However, training before tests on animals and control of the applying force by reading the value indicated on the display can contribute to reduce variability of the mechanical waveform between tests. Similarity between scores obtained by 2 investigators suggests that the low variability of mechanical waveform has limited influence on pain threshold values. Because requirement for reliability in behavioral models of nociception was not based on mechanical reproducibility but on consistency of scores within repeated stimulus,17 we can consider that our device meets this criterion.

**Mechanical Hyperalgesia Induced by Carrageenan**

The present report shows that results obtained with forceps are in accordance with the literature on Randall-Selitto tests or von Frey filaments.2,11 Because of the stress of the procedure, Randall-Selitto tests can show high variability and thus necessitate a great number of rats (≥6) and intensive training to reach significant differences between groups.1,29 Our study suggests that these difficulties are less present with the calibrated forceps, so that it is possible to design studies with fewer rats per group, rapidly trained before experimental onset.

We also performed von Frey filament tests on freely moving rats maintained in Plexiglas boxes. Despite the absence of contention stress, our results with von Frey filaments were less significant than with forceps. This difference might be due to the use of a low number of animals, which was done to keep conditions similar to the forceps experiment, and to the difficulty to determine whether leg movements were due to pain reflex or to the filament pushing the paw.27 Furthermore, the necessity to use several filaments (at least 3) to determine pain threshold might lead to poor precision because of the logarithmic and discontinuous scale of the filament set.

It should also be stressed that the 2 models did not exactly generate identical stimulations. Calibrated forceps and von Frey filaments readings differ by a factor of \(\sim 2\) (Fig 3, B and C), which mainly results from the different shapes of the devices. Von Frey filaments apply a force on a very small area of the plantar paw (eg, 0.42 mm² for von Frey filament 5.88)18,25; the calibrated forceps produce a pinch effect caused by forces applied to a larger area (\(\sim 10\) mm²) on both sides of the paw. Because pressure is the actual stimulus applied to the paw, a correspondingly larger force must be applied to produce the same result. However, residual discrepancies between the 2 stimulation types might also result in the
activation of different receptor fields in the spinal cord and/or supraspinal structures.

**Analgesia and Tolerance to Morphine**

Analgesia and tolerance to morphine were studied to check whether it is possible to reproduce known results with the calibrated forceps. In the present study this device detected morphine analgesia even by using morphine at low concentration (2.5 mg/kg). The capacity to detect analgesia at this low dose is in accordance with previous results by using hot plate and tail immersion tests with Wistar rats. When doing repeated exposures to morphine (10 mg/kg), we observed progressive tolerance to the analgesic properties of this opiate. Several articles describing opiate tolerance have proposed this tolerance to be only apparent and to result from a delayed and progressive decrease of the basal nociceptive threshold to mechanical or thermal stimulation after opiate injections. However, in the present study, the once-daily morphine injection during a period of 5 days did not affect basal mechanical threshold. Our protocol settings (rat strain, injection route, injection schedule, and nociceptive tests) are unlikely to explain this discrepancy with literature. Consequently, the type of opiate agonist and/or the dose injected are the most likely explanation for this apparent discrepancy. Indeed, hyperalgesia was generally shown with heroin or fentanyl rather than morphine. Two studies showed that intrathecal or intravenous morphine can also induce hyperalgesia; however, this effect might be due to the administration route. Indeed one of these studies showed that the intravenous route induced hyperalgesia, whereas the subcutaneous route (similar to our present results) was inefficient. Taken together, these results suggest that high opiate efficiency might be needed to induce long-term hyperalgesia; hence it is possible that in our model the efficiency was too weak to induce this effect. Opiate tolerance was generally determined by comparison before and after chronic treatment. In contrast to this procedure, our experimental schedule, which planned daily pre-injection and post-injection tests, represents a very accurate control to measure the analgesic effect of morphine. This protocol is allowed by the stability of the measures obtained with the calibrated forceps. This feature increases the opportunity to detect not only acute but also long-lasting analgesic effects of a drug, as well as potential changes in basal nociceptive threshold consecutive to a chronic treatment. Taken together, these results suggest that it might be possible to discriminate easily between opiate-induced hyperalgesia and tolerance by using our experimental protocol.

In conclusion, our study shows that calibrated forceps are convenient to perform study of paw mechanical nociceptive threshold in rats. Development is currently ongoing to possibly adapt this tool to studies of mouse nociceptive threshold. This tool is easy to implement, allows rapid testing of the animals, and meets criteria for nociceptive measurement: sensitivity, reliability, and reproducibility.

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