Chapter 14. Experimental models for the evaluation of somatic pain in rodents

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There are more than 10,000 conditions in human health that are accompanied by pain, and while the evaluation of pain in humans is, most of the times, a straightforward method, with the subject being asked and responding about the perceived pain level, the evaluation of pain in test animals is a more challenging enterprise. In this case, the method should be adapted and the experimenter must find other ways for the interpretation of the animal “language” or physical response to pain and its quantification. To add to the level of complexity, there are different types of pain that can be defined and, of course, evaluated: acute or chronic; superficial or deep; somatic, visceral or central, etc.

Since the early XXth century various objective measures have been developed to quantify nociception and analgesic responses using non-human models. While each of the tests had strengths and weaknesses from a research perspective, several have become outdated due to modern ethical concerns.

The purpose of this chapter is a short review of the most common experimental rodents models for the evaluation of pain, currently in use in our Centre for the Study and
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Therapy of Pain. All experimental procedures described are consistent with the international ethical regulations and detailed in the ethical issues chapter. A special note to all the methods presented is that, as in all behavioral testing models, animals should undergo several episodes of adaptation to handling and exposure to the test environment, including transportation of the animal to the test room, placing the animal in the test device, and returning the animal to the home cage. This better minimizes the false positive or false negative responses of the animals due to changes in their environment and not necessarily related to the test itself. Also, all experiments should be performed at the same time of the day between 9:00 a.m. and 3:00 p.m. to avoid diurnal variation in behavioral tests.

1. Central Pain Evaluation

1.1. Mechanoalgezic Test - Analgesimeter Test

*Principle*

The test is performed in rats using an analgesimeter (model 7200, Ugo Basile, Varese, Italy)-Figure 1. Linearly increasing down force (16 g/s) is applied on the hind paw, between the third and fourth metatarsal. The maximum threshold (cut-off limit) in the literature is 15 units. The force threshold will not be exceeded during the experiments, though a smaller cut off limit may be set depending on various factors. The value obtained for complete paw withdrawal will be noted down.

At the beginning of each experiment the device is calibrated and the animal lot tested to a certain homogeneity level with
unresponsive or aberrant animals being removed. Control group values are noted and compared with the test values. The animals are held vertically without causing any discomfort and the metatarsi three to four from the right hind paw is placed on the special Teflon holder. A tapered rod with a rounded tip is lowered gently on the paw so as not to produce any stress. The operator presses the analgesimeter pedal and increases the applied force and as such increases the pressure of the rounded cone at the tarsal level with about 16 g/s. When the animal feels pain it reacts by withdrawing the paw and/or whining, and the operator stops the device, while noting the value of the cursor, considered to be the threshold of pain.

Any animal with a control pain threshold greater than 80 g is eliminated and replaced. For a certain time response, groups of at least 7 animals are used. Evaluation for changes in pain threshold is done at 15 minutes intervals after subcutaneous administration and at 30 minutes intervals after oral

![Analgesimeter Ugo Basile](image)
administration. The interval of time, which indicates the greatest increase in pain threshold, is regarded as the peak time. A dose range is obtained in the same manner as the time response. The drug to be tested is administered in a randomized manner. The pain threshold is recorded at time zero and again at the determined peak time. The mean applied force is determined for each time interval tested. Latency is determined once for each animal at one time interval.

If the test is performed in inflammatory pain, (e.g. induced with carrageenan), the latency time is determined for the basal (baseline, pre-carrageenan) and 2h and 30 minutes after injection of carrageenan. After the administration of the tested drugs, like analgesics, the latency time is measured again at 10, 20, 30 and 40 minutes (time post - dose latency). Treatments that produce a significant increase in nociceptive threshold are considered to be antinociceptive. The results are compared with the control group.

The percentage increase in pain threshold is calculated with the formula:

\[
\text{% increase in pain threshold} = \frac{F_t - F_c}{F_c} \times 100
\]

where

\(F_c = \text{mean applied force in control group}\)
\(F_t = \text{mean applied force in test group}\)

This method was originally described by Randall and Selitto and has been used by many researchers, as it has been
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proven to detect central analgesics as well as peripheral analgesics. Peripherally acting analgesics such as the nonsteroidal anti-inflammatory drugs increase only the threshold of the inflamed paw, whereas opiate analgesics increase also the threshold of the intact paw. In most later modifications, the assay has a shallow dose-response curve. Nevertheless, the ED50 values of nonsteroidal anti-inflammatory drugs in this test showed a good correlation with human doses.

Advantages of the method:
- Constant force can be increased;
- Cursor stops when the pedal is not reached;
- Pressure point is fixed;
- Does not require anesthesia.

Disadvantages
- Not relevant if the animal reacts to discomfort or pain;
- Not enough time to accommodate before testing;
- Error can occur during data acquisition;
- Two persons are required to do the test.

1.2. Thermoalgezic Tests

- Plantar Test

Principle
The method is based on the animal’s reaction to a source of heat of variable intensity. Rats accommodate with the aperture for five to ten minutes before testing. The device consists of a restraining device with six individual transparent plastic cages placed on a glass substrate. A
mobile radiant heat source is located under the support and placed at the desired paw - in this case, in the right hind paw. The infrared source is connected via a timer circuit, starting with the onset and reducing the energy source simultaneously with the movement made by the animal tested. As soon as the animal reacts by raising paw, licking it or any change of position, the timer stops recording. The radiant energy source is applied to the test animal in the right hind paw and the operator waits for rats’ reaction – the recorded paw withdrawal time (lag time, PWT) by using a timer. The maximum test (cut-off time) is set to 40 seconds and stops automatically when the rat paw is raised.

For inflammatory pain evaluation, the latency is determined once for each rat, at each recording part: basal (base-line, pre-carrageenan) and 2h and 30 minutes after injection of carrageenan. After administration of tested drugs, like analgesics, the latency time is measured again at 10, 20, 30 and 40 minutes (time post-dose latency PWT). Treatments that produce a significant increase in nociceptive threshold are considered to be antinociceptive. The device for the plantar test and the reaction found during the test can be seen in Figure 2.
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**Advantages:**
- Data recordings are digital;
- Easy to use.

**Disadvantages:**
- The moment in which the rodent is sniffing, licking its forepaws and hind paws, cannot be seen by the examination operator - human error;
- The test is very susceptible to learning phenomenon.

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*Fig. 2. Plantar test setting*
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- Hot Plate Test

Introduction
The Hot Plate is the most commonly used test to study thermal (anti)nociception in rats and mice and is based on the fact that the paws of the mice and rats are very sensitive to heat, at temperatures which are not damaging the skin. In this test, the animal is placed on a hot plate until a predetermined behavioral endpoint is observed, typically a paw lick, paws withdrawal or jump response. Differences in latencies between animals are considered to reflect differences in nociceptive threshold. It is known that when studying drug-induced changes in nociceptive threshold, latencies of various behavioral endpoints are affected differently depending on the drugs used, therefore the time until these responses occur is prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses. The method was described originally by Woolfe and MacDonald in 1944 and has been modified by several investigators for specific applications.

Materials and method
Separate groups of mice are used for each dose, which is previously labeled. The animals are transported to the room and left undisturbed for at least 15 minutes before the test (assuming the conditions in the testing room are the same as in the rest of the facility – if they are not, allow 1 hour for acclimatization). The hot plate is heated at a temperature of 54±0.5°C and the time until the end point is measured by a stopwatch. The end points are considered when the animal first licks one of its rear paws (front paw licking is a common
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grooming response and may have no relation to discomfort therefore, rear paw licking is a more reliable measure of discomfort), attempting to escape, shaking hind paw or vocalization.

The duration of action of a compound may also be determined by measuring a single animal's response over time, thus measurements may be done 15, 30, 45 and 60 min or even longer after treatment. Of course, similar measurements must be done for the control group.

The experimental values can be used for statistical comparison using the “t-test”, or, alternatively, the values, which exceed the one before administration for 50% or
100%, can be regarded as positive and ED50 values can be calculated. Data from hot-plate tests are also often expressed as percent maximum possible effect (%MPE).

\[
\text{% MPE} = \frac{\text{test} - \text{baseline}}{\text{cutoff} - \text{baseline}} \times 100
\]

where

Test = the latency to respond after treatment
Baseline = the latency to respond prior to treatment
Cut off = is the preset time at which the test will be ended in the absence of a response

To determine a compound’s % MPE, a baseline measure must be obtained for each animal prior to treatment with control or test compound.

**Advantages / Disadvantages:**
The hot plate test has been used by many investigators and has been found to be suitable for evaluation of centrally but not of peripherally acting analgesics. Mice as well as rats have been used but it was noted that behavioral responses are more complex in rats than in mice. Rats can react with sniffing, licking its forepaws and hind paws, they straighten up, stamp their feet, start and stop washing itself. There are examples of unlearned behaviors more complex than reflexive responses and they appear to represent higher cerebral functioning.

It should be taken into consideration that this is a method of evaluating analgesic activity of central origin, therefore peripherally acting analgesics show little or even no activities
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in this test. The method has the drawback that sedatives and muscle relaxants or psychotomimetics cause false positive effects, while mixed opioid agonists-antagonists provide unreliable results. As far as analgesic substances are concerned, the paw-licking behavior is affected only by opioids but on the other hand, the jumping reaction time is increased equally by less powerful analgesics, such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50°C or less.

Likewise, this test is very susceptible to learning phenomenon, which results in a progressive shortening of the jumping reaction time, accompanied by the disappearance of the licking behavior. It triggers shortening the reaction time even by placing the animals on an unheated plate just once a day or once a week, and leads to a decrease in the reaction time.

- Tail-Flick Test

Introduction
The tail flick test was developed by D’Amour and Smith in 1941 and remains a commonly used test for the assessment of analgesia. Originally the method was developed for quantitative measurements of pain threshold against thermal radiation and for evaluation of analgesic activity of opioids. Later on, the procedure has been used by many authors to evaluate analgesic activity in animal experiments, by measuring drug-induced changes in the sensitivity of mice or rats exposed to heat stress applied to their tails. The test is very useful for discriminating between centrally acting morphine-like analgesics and non-opiate analgesics. This
test is easy to learn, simple to conduct with minimal equipment and produce only a transitory thermal pain that can be repeatedly applied to individual animals.

**Materials and Methods**

The Tail Flick Unit (Ugo Basile, Varese, Italy). The used animals are 20±2 g male mice or 200±20 g male rats. Before the experiment the animals are weighted, labeled and left 15 minutes to accommodate to the test environment before the determination. Separate groups of animals are used for each substance.

First the baseline of latency of each animal is recorded; the intensity of heat source is adjusted to produce tail-flick latencies in the range of 3 to 4 seconds. The animals are placed into cages, leaving the tail exposed to the light beam, which is focused to the distal third of the tail on the dorsal side. Within a few seconds the animal flicks the tail aside or tries to escape and the time until this reaction occurs is measured. In the absence of a withdrawal reflex, the stimulus cutoff time is set to 10 seconds to avoid possible tissue damage. Mice with a reaction time over the cutoff value are not used in test. To improve accuracy, three separate determinations of tail-flick latency per animal are made, and mean is calculated. This mean will represent the baseline latency for the animal. The animals may be tested in 3 to 5-min intervals. To avoid tissue damage, the stimulated area will be varied 10 mm in both directions away from the original site for the second and third stimulation.

The animals are injected with the test agent, vehicle control, or reference compound and then tested again at 15, 30, 60
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minutes etc. The time for the animal to show a tail-flick response is recorded, or assigned a value of 10 sec (cutoff time) if no tail-flick is observed. Only one post-drug tail-flick test at each time point is performed. If the time-course of effect is known for the agent, tail-flick testing should be performed at the peak effect; testing at one time point helps minimize tissue damage to the tail.

Data generated using the tail-flick test is often expressed as the percentage of maximal possible effect (%MPE):

% MPE = (post injection latency - baseline latency) / (cutoff - baseline latency) \times 100
To determine an ED50 value (dose that produces 50% effect), the data must be expressed as % effect.

**Advantages / Disadvantages**
The escape reaction, which is the endpoint of this test, can be regarded as a complex phenomenon mediated by the brain. In contrast, the simple tail flick as an endpoint of this test may be mediated as a spinal reflex. Therefore the observation of the escape reaction can be regarded as a true assessment of the influence of the drug on the brain.

2. Peripheral Pain Evaluation

2.1. Inflammatory Pain Evaluation

**Introduction**
Inflammation has long been known to cause pain, edema and pyrexia, all of which are reduced by salicylates. A method for the measurement of analgesic activity on inflamed tissue was first reported by Randall & Selitto in 1957, who made use of the knowledge that inflammation increases sensitivity to pain and that this increased sensitivity can be influenced by analgesics.

The inflammation is produced by the injection of a dry yeast suspension into the plantar tissue of rodent hind paw and the effectiveness of analgesic drugs is determined by measuring the pressure (initially in mmHg, nowadays in grams) to which the paw can be subjected before the rat exhibits a flight (struggle) response. The inflamed paw is about twice as sensitive to pressure-induced pain as the non-inflamed paw, and narcotic as well as mild analgesics are
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capable of elevating the reaction threshold in a dose-dependent manner.

Materials and Methods
Wistar rats are typically used, with a weight between 200±20 g at the beginning of the experiments. The route of administration can be intraperitoneal or subcutaneous. For oral administration only, the animals are fasted for 18 to 24 hours prior to the test.

Fig. 5. Rodent adjustment to climate/cage

- Carrageenan model of inflammatory hyperalgesia

This method was first described in 1991 by Wheeler-Aceto and Cowan. This method is used to study the action of opioid drugs with analgesic effects in the periphery. If in the past it was known that opioid receptors are only found in the
spinal ganglia and the central level, more recently their existence was demonstrated also in the periphery, the peripheral sensory nerve endings in rats and humans. It has been shown too that these receptors are stimulated during inflammation, which is why we choose to model peripheral carrageenan inflammation.

The rats receive an injection of 0.1 ml \( \lambda \)-carrageenan 1%, diluted in 0.9% saline solution at the back right paw. Other substances can be used to induce inflammation such as 0.1 ml of a 20% suspension of Brewer’s yeast in distilled water injected subcutaneously into the plantar surface of the hind paw of the rat; Freund’s adjuvant or prostaglandin E2. The animals in the described experiment develop local inflammation, which remains confined in the injected paw. For the study of opioid peptides in acute inflammation we administrate the tested opioid at 2 hours and 40 minutes after injection of carrageenan. As a parameter of inflammation the paw volume plethysmometer is measured.

To assess inflammatory hyperalgesia arising from the injection of carrageenan, two nociceptive tests (presented above) can be performed:
- mechanical nociceptive (analgezimeter test)
- nociceptive thermal (plantar test).

**Advantages of the method:**
- Requires no control group, the not affected paw being used for this purpose;
- Ease of technique
- Local reactions are obtained
**Disadvantages of the method:**
- Requires plethysmometer to quantify inflammation;
- Reading error may be present.

**Evaluation of inflammatory edema in carrageenan - plethysmometric test.**

**Principle**
This test allows the measurement of the volume of the hind paw and is used to evaluate the inflammatory effect of drugs in acute and chronic inflammation. A model 7140 Ugo Basile plethysmometer is used. The method was originally described by Rendall - Sellito. The reference medicine is
phenylbutazone at a dose of 80 mg/kc, dissolved in 1% methylcellulose solution. At the beginning of the experiment, the unit is cleaned so Perspex cells of various diameters should be permeable, filled with fresh solution to the limit and then calibrated. A transducer records tiny changes in water volume displaced by introduction of the tested animal paw. The device indicates exactly the volume of the studied paw. If inflammation is noticeable, there is an increase in volume over time, and the result is compared with basal recordings. Paw volume is measured once for each rat. The value is determined at the time of the baseline (baseline pre-carrageenan), and then at 2 hours, and 30 minutes after injection of carrageenan. After administration of test drugs, the value obtained is noted and every 3 hours, 3 hours and 30 minutes respectively after injection of carrageenan the paw volumes are measured (volume plantar post-dose).

Advantages:
- The inflammation is quantifiable;
- Data recordings are digital.

Disadvantages:
- Level marked on the paws could not be the same;
- Depth in which the paw is introduced can be different;
- The moment in which the measurement of inflammation is done may be not the same, human error.
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Fig. 7. Plethysmometer Ugo Basile

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