Algynomics Preclinical Pain Service – Technical Details

1.0. General Considerations

1.1. Subject Choice

The Mogil laboratory has, over the years, documented the important role of genotype [13,23-24] in affecting both basal nociceptive sensitivity, the efficacy of analgesics [29,31], and the interpretation of null mutant phenotypes [12]. In addition, the Mogil laboratory has studied the important and still neglected issue of sex differences [15], and demonstrated robust *qualitative* sex differences in the operation of pain circuitry [4-6,20,22,25-26].

Although clients are free to specify subject genotype and sex, we strongly recommend consultation on this issue.

1.2. Testing Environment

A major difference between the Mogil laboratory and other pain testing facilities in academe and industry is our attention to organismic and environmental modulatory factors that can radically alter apparent nociceptive sensitivity. We have published papers on the previously unappreciated role in pain of injection stress [19], arousal at the moment of testing [1], experimenter effects [3], seasonal variation [2], and visual observation of neighbors in pain ("empathy") [9-11]. We have instituted a number of changes in our experimental setups and testing paradigms in order to turn these observations into "best practices" not used elsewhere.

1.3. Sample Sizes

In our extensive experience running these assays, it requires approximately n=12 mice/genotype or mice/drug dose to evince differences on Acute Assays, but the same n=12 mice can be used for all of them. For the Tonic Assays, n=12/group are also required, but different mice are needed for each assay. For Chronic Assays, n=12–16 mice/group are usually required. These sample sizes are higher than those commonly used (n=8–9) [21], but too-low sample size is a common reason for non-replicable effects.

1.4. Housing, Husbandry and Habituation

Mice are bred in-house, received from the client, or purchased from commercial suppliers (The Jackson Laboratory, Charles River Quebec, Taconic Farms). If bred in-house, they will be housed with same-sex littermates following weaning at 18–21 days, with no more than 4 males/cage or 5 females/cage. If purchased or received, they will be housed 4 mice per same-sex cage. Mice are fed, watered and given environmental enrichment as per McGill University regulations. In every case, experiments will not commence until mice are at least 6 weeks of age, and have been living in the "working vivarium" (directly across the hall from the testing suite) for at least 1 week. On each day of testing, mice will be habituated either to the testing room and/or to the Plexiglas observation cylinders or cubicles for at least 30 min. In assays requiring non-moving mice (see sections 2.1.4–2.1.6), more extensive (>2 h) habituation is required.

1.5. Behavioral Testing Suite

The Mogil laboratory at McGill University contains a behavioral testing suite that is likely the most advanced in the world. Comprising over 800 square feet in four separate rooms (three

private), it contains not only all the necessary commercial equipment to run these assays, but the rooms have been ergonomically designed for maximum throughput, tester comfort, noise and distraction abatement, and the lowest possible stress levels for murine subjects.

2.0. Description of Assays

2.1. Acute Assays

2.1.1. Hot-Plate Test

This test, developed in 1944 by Woolfe & McDonaldinvolves placing the subject on a metal surface maintained at a temperature above the nociceptive threshold (about 49ºC). The animal's movement is constrained by Plexiglas walls. Escape is possible by jumping off the plate, but in naive mice more common indices of nociception are either licking of the hindpaw or rapid fluttering/shaking of the hindpaw. After any of these responses are observed by an experimenter, the mouse is immediately removed from the plate (taking no more than 2–3 s) and the latency recorded. We use 50–56 ºC as the hot-plate temperature. In the absence of any nociceptive responses (in an animal pretreated with an analgesic, or in an insensitive mouse strain) after 60 s (or three times baseline latency, whichever comes first), the animal is removed from the plate to avoid any possibility whatsoever of tissue damage.

2.1.2. Tail-Clip Test

This test, developed by Haffner and modified by Takagi et al. in 1966, involves gently restraining the mouse, applying a binder clip (500-800 g of force) appoximately 1 cm from the base of the tail, and then removing the mouse from the restraint. The latency to attack/bite/lick the clip is measured with a stopwatch, and the clip is then immediately removed(taking no more than 5 s). A cut-off latency of 60 s is imposed to prevent the possibility of tissue damage.

2.1.3. Tail-Withdrawal Test

This test is a modified form of the classic tail-flick test developed in 1941 by D'Amour & Smith. In the popular version to be used, the distal half of the animal's tail is immersed in hot water maintained at 46–53 ºC, and the time taken to vigorous reflex withdrawal of the tail is measured. The "cut-off" time for this test in the absence of a withdrawal is 15 s. We use 46–49 ºC as the water temperature. The classic tail-flick test can be substituted if desired.

2.1.4. Radiant Heat Paw-Withdrawal Test

This test was developed in 1988 by Hargreaves and colleagues. For testing the hind or fore paws the mouse is placed on an elevated glass floor within Plexiglas cubicles(7 x 6 x 11 cm). A radiant heat source with a 3x5 mm aperture is placed beneath the glass floor and the light is aimed at the plantar surface of the paw. The timer yields the latency to the mouse's withdrawal from the stimulus (by hopping away) with an accuracy of 0.1 sec. An automatic cut-off of 40 s is programmed to prevent tissue damage in the absence of a response. At a given time point, each paw is tested up to 3 times with at least 2 min between successive tests.

2.1.5. von Frey Filament Test

The mouse is placed on an elevated wire mesh grid within Plexiglas cubicles (7 x 6 x 11 cm). In the "manual" version of the test, the plantar surface of the hindpaws are gently poked with calibrated von Frey filaments (≈0.001–2 g) until the filaments bow for 1–5 s. Whether the mouse withdraws from the stimulus (by hopping away) or not is recorded. An up-down psychophysical method is employed to determine the mechanical threshold of each paw. Note that at and above 2 g application of the fiber simply lifts the hind paw in the air, without bowing the fiber. In the "automated" version of the test, a single motor-driven fiber increases the pressure until the mouse withdraws, and the unit displays the maximum pressure emitted. Typically, baseline withdrawals occur at approximately 6 g of force; the machine is programmed to cut-off at 10 g. With both versions, at a given time point, each paw is tested up to 3 times with at least 2 min between successive tests. Mice can be tested with either or both of these methods.

2.1.6. Cold Sensitivity (Acetone)Test

The mouse is placed on an elevated wire mesh gridwithin Plexiglas cubicles (7 x 6 x 11 cm). A syringe is fitted with a small bore needle and filled with 0.15 ml of acetone. The needle is aimed at the plantar heel (without touching the skin) and the acetone is squirted onto the heel. Evaporative cooling creates a stimulus of approximately 10 ºC that lasts for a second or two. When applied to human skin this stimulus evokes a distinctly cold but non-painful sensation. This test is performed once on each hind paw and the duration of the withdrawal response(along with any lifting/shaking behaviors) is measured with a stopwatch.

2.2. Tonic Assays

2.2.1. Abdominal Constriction ("Writhing") Test

This test is a classic assay of reflexive response to a visceral/muscle wall irritant (Koster, 1959). Acetic acid (0.1–1.2%) is injected intraperitoneally (10 ml/kg), and the number of lengthwise constrictions of the abdominal musculature are counted for 30 min. In some cases, acetic acid can be substituted for by the non-inflammation-producing magnesium sulfate (120 mg/kg), which produces similar abdominal constrictions but for 5–10 min only.

2.2.2. Formalin Test

This test of biphasic (acute and tonic) nociception was developed, at McGill, in 1977 by Dubuisson and Dennis. The test involves the subcutaneous injectionof a dilute solution of formalin (itself a dilute 37% weight/weight solution of formaldehyde [H2=C=O]) into the plantar hind paw. We use common parameters for mice: 25 µl of a 1–5% solution (dissolved in saline). Of the variety of known tissue irritants—acetic acid, carrageenan, kaolin, platelet-activating factor, mustard oil, serotonin, yeast—formalin produces the most reproducible behavioral response, consisting in mice of licking/biting the injected paw and surrounding hindquarters. The response is biphasic, with "acute" or "early" phase responding 0-10 min after injection, a quiescent period from 10-20 min, and then a "tonic" or "late" phase occurring 20-60 min after injection. Formalin can be substituted for by 5% allyl isothiocynate (mustard oil) or *Apis mellifera* (honey bee) venom (0.1–0.3 mg/paw), both producing similar behaviours (but with a monophasic time course) lasting <60 min.

2.2.3. Capsaicin Test

Injection of capsaicin (1–5 μl) into the plantar hind paw produces monophasic licking/biting of the injected paw similar to formalin, but with a greatly reduced duration of approximately 10–15 min. It is performed as described in section 2.2.2 above. It has the advantage of being used in humans, and thus we often use this assay to assess whether a human study should also be performed.

2.2.4. Postoperative Pain Test

First developed by Brennan and colleagues in 1996, it has been demonstrated that surgical incisions, under anesthesia, lead to hypersensitivity of the incised skin lasting up to a week.Weperform one of two models: 1) either a <1–cm incision of the plantar hind paw, or 2) a sham ovariectomy (ventral approach; i.e., a laparotomy). Mechanical hypersensitivity of the hind paw or abdomen is measured on days 1, 2, 4, and 7 postoperative using the von Frey test as described in section 2.1.5.

2.3. Chronic Assays

2.3.1. Surgical Nerve Injury Assays

Mice undergoing the neuropathic surgeries described belowwill be tested in one or more of the behavioral tests described in section 2.1.4–2.1.6. Tests of pre-injury (normal) baseline sensitivity (see below) are performed 1–3 times prior to treatment on different days. Tests of post-injury sensitivity begin on postoperative day 1, and continue on postoperative days 4, 7, 14, 21 and 28.

2.3.1.a. Chronic Constriction Injury.Under isoflurane/oxygen anesthesia, the common sciatic nerve is exposed via a skin incision ≈7–mm long on the dorsal thigh and blunt dissection through biceps femoris. Using an operating microscope (x40), the nerve is freed of adhering fascia, taking care to avoid stretching the nerve. On the experimental side, 4 ligatures (chromic gut) are tied loosely around the nerve. The ligatures are tied loosely such that the diameter of the nerve is just barely reduced. These loose ligatures impede blood flow through the epineurial capillaries and this causes endoneurial edema. During the first 24–48 h after surgery, the pressure from the increased fluid is opposed by the ligatures; and the nerve self strangulates. This creates a chronic constriction injury that interrupts almost all of the large myelinated axons and a large majority of the small myelinated axons, but spares most of the unmyelinated axons.

2.3.1.b. Spared Nerve Injury. Under isoflurane/oxygen anesthesia, and using an operating microscope (x40), the three terminal branches of the sciatic nerve (tibial, sural and common peroneal) are exposed. The tibial and common peroneal nerve are cut, after tight ligation with 6.0 silk), "sparing" the sural nerve. Because the sural nerve is spared, when using this assay von Frey filaments are aimed (both at baseline and postoperatively) at the sural territory, not the mid-plantar region.

2.3.1.c. Spinal Nerve Ligation. Under isoflurane/oxygen anesthesia, and using an operating microscope (x40), the L4, L5 and L6 spinal nerves are exposed. The L5 and L6 nerves are tightly ligated, sparing the L4 spinal nerve.

2.3.2. Chronic Inflammatory Assays

Mice undergoing the inflammatory assays described below will be tested in one or more of the behavioral tests described in section 2.1.4–2.1.6. Tests of pre-injury (normal) baseline sensitivity (see below) are performed 1–3 times prior to treatment on different days. Tests of post-injury sensitivity depend on the known duration of hypersensitivity after each inflammatory mediator, and will be noted below. In our hands, at these concentrations of all three algogens, no evidence of spontaneous nociception (e.g., licking, biting) is seen; the compounds simply lower the threshold to respond to thermal (heat and cold) and mechanical stimuli.

2.3.2.a. Zymosan. Intraplantarzymosan A (from *S. cerevisiae*) produces hypersensitivity peaking at approximately 4 h post-injection and resolving by 72 h post-injection. We will inject mice subcutaneously in the plantar hind paw with 0.25–2.0 mg/ml zymosan. Post-injection testing will occur at 1, 2, 4, 12, 24, 48 and 72 h.

*2.3.2.b. Carrageenan.*Intraplantar carrageenan produces hypersensitivity peaking at approximately 4 h post-injection and resolving by 72 h post-injection. We will inject mice subcutaneously in the plantar hind paw with 1–2% carrageenan. Post-injection testing will occur at 1, 2, 4, 12, 24, 48 and 72 h.

2.3.2.c. Complete Freund's Adjuvant (CFA). Intraplantar CFA produces hypersensitivity peaking at approximately 3 days post-injection and lasting for up to 2 weeks. We will inject mice subcutaneously in the plantar hind paw with 50% CFA (0.5–2.0 mg/ml). Post-injection testing will occur at 1, 2, 4, 7, 10, and 14 days.

2.3.2.d. Lipopolysaccharide (LPS). LPS is known to produce hypersensitivity by all routes of administration: systemic, intraplantar, intrathecal, and intracerebroventricular. We will administer LPS in the following dose range for each route: systemic (1-5 mg/kg), intraplantar (1–10 μg), intrathecal (0.01–1 μg), intracerebroventricular (1–10 μg). At the high range, these doses are associated with hypothermia (<4 ºC), but not lethality. By any route, LPS produces hypersensitivity peaking at approximately 4 h post-injection and resolving by 24–48 h post-injection. Post-injection testing will occur at 1, 2, 4, 12, 24 and 48 h.

2.4. New Assays

Dr. Mogil spends much of his academic efforts staying abreast of developments in the field of algesiometry. We attempt to replicate recently developed assays, and are constantly developing our own. For example, we can perform medial meniscus destabilization surgery (a model of osteoarthritis), and have developed behavioral assays of vulvodynia and migraine. Consult with Dr. Mogil if you are interested in a novel assay.

2.5. New Measures

Most chronic assays feature the measurement of mechanical and thermal hypersensitivity, even though it is widely appreciated that this is a suboptimal strategy. A number of new measures of spontaneous pain have been recently proposed. Once we are convinced that ameasure is useful (in our own hands; not simply because a paper has been published!), we will master it and offer it to clients.

2.5.1. Facial Expression of Pain (The Mouse Grimace Scale)

As recently reported [8], we adapted the human facial pain coding scale (FPCS) for use in the mouse. The Mouse Grimace Scale (MGS) has five facial "action units": orbital tightening, nose bulge, cheek bulge, ear position, and whisker change. Mice are videoed at baseline and after the noxious stimulus; still photographs are grabbed from the video files, randomized, and scored on each action unit. Pain is quantified as the mean difference score between "pain" and "baseline" photographs. The following assays were reported to be associated with a "pain face": magnesium sulfate, acetic acid, formalin test (late phase), mustard oil, hind paw incision, laparotomy, zymosan (hind paw and ankle), and cyclophosphamide cystitis. The MGS is decreased by ibotenic acid lesions of the rostral anterior insula, suggesting that it is measuring pain affect. Although the implementation of the MGS is labor-intensive, we have recently developed software designed to automate the photo grabbing, the most labor-intensive part of the process. This has greatly decreased the time requirement to perform MGS testing.

We have recently been adapting the MGS to the rat. A rat "pain face" has been demonstrated after complete Freund's adjuvant, laparotomy, and kaolin/carrageenan injection (unpublished data).

2.5.2. Conditioned Place Preference

As reported by Sufka and colleagues in 1994 [28], and rediscovered by King et al. in 2009 [7], inhibition of chronic pain by analgesics can represent an unconditioned stimulus (UCS) leading to Pavlovian conditioning to the environment where that pain inhibition occurred. We can perform such experiments using a photocell-based conditioned place preference arena. Conditioned place aversion to pain itself can also be used.

2.5.3. Operant Paradigms

A variety of operant paradigms have been developed in which a rodent subject is trained to avoid painful stimuli via escaping from the stimulus. In some cases, motivational conflicts between pain and rewarding stimuli are utilized. Clients are advised to consult with Dr. Mogil about which of a large number of options can and should be utilized in this domain.

3.0. References

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